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(54) Title: PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) Abstract: This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

Production of Humanized Antibodies In Transgenic Animals

5 Field of the Invention

This invention relates to humanized antibodies produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

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Background of the Invention

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. Therapies of patients with malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high. For both infectious diseases and cancer, improved and innovative therapies are needed.

Therapy of steroid resistant rejection of transplanted organs requires the use of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing 20 alloimmune response in the transplant recipient. The major problem of antibody preparations obtained from animals is the intrinsic immunogenicity of non-human immunoglobulins in human patients. In order to reduce the immunogenicity of non-human antibodies, genetic engineering of individual antibody genes in animals has been proposed. In particular, it has been shown that by fusing animal variable (V) region exons 25 with human constant (C) region exons, a chimeric antibody gene can be obtained. However, this approach may only eliminate the immunogenicity caused by the non-human 30

Fc region, while the remaining non-human Fab sequences may still be immunogenic. In another approach, human immunoglobulin genes for both, heavy and light chain immunoglobulins have been introduced into the genome of mice. While this genetic engineering approach resulted in the expression of human immunoglobulin polypeptides 5 in genetically engineered mice, the level of human immunoglobulin expression is low. This may be due to species-specific regulatory elements in the immunoglobulin loci that are necessary for efficient expression of immunoglobulins. As demonstrated in transfected cell lines, regulatory elements present in human immunoglobulin genes may not function properly in non-human animals.

10 Several regulatory elements in immunoglobulin genes have been described. Of particular importance are enhancers downstream (3') of heavy chain constant regions and intronic enhancers in light chain genes. In addition, other, yet to be identified, control elements may be present in immunoglobulin genes. Studies in mice have shown that the membrane and cytoplasmic tail of the membrane form of immunoglobulin molecules play 15 an important role in expression levels of human-mouse chimeric antibodies in the serum of mice homozygous for the human *Cy1* gene. Therefore, for the expression of heterologous immunoglobulin genes in animals it is desirable to replace sequences that contain enhancer elements and exons encoding transmembrane (M1 exon) and cytoplasmic tail (M2 exon) with sequences that are normally found in the animal in 20 similar positions.

The introduction of human immunoglobulin genes into the genome of mice resulted in expression of a diversified human antibody repertoire in genetically engineered mice. In both mice and humans, antibody diversity is generated by gene rearrangement. This process results in the generation of many different recombined V(D)J segments 25 encoding a large number of antibody molecules with different antigen binding sites. However, in other animals, like rabbits, pigs, cows and birds, antibody diversity is generated by a substantially different mechanism called gene conversion. For example, it is well established that in rabbit and chicken, VDJ rearrangement is very limited (almost 90% of immunoglobulin is generated with the 3' proximal VH1 element) and antibody 30 diversity is generated by gene conversion and hypermutation. In contrast, mouse and

human gene conversion occurs very rarely, if at all. Therefore, it is expected that in animals that diversify antibodies by gene conversion a genetic engineering approach based on gene rearrangement will result in animals with low antibody titers and limited antibody diversity. Thus, the genetic engineering of large animals for the production of non-
5 immunogenic antibody preparations for human therapy requires alternative genetic engineering strategies.

Relevant Literature

The use of polyclonal antibody preparations for the treatment of transplant
10 rejection was recently reviewed by N. Bonnefoy-Berard et al., *J Heart Lung Transplant* 1996; 15(5): 435-442; C. Colby et al., *Ann Pharmacother* 1996; 30(10):1164-1174; M.J. Dugan et al., *Ann Hematol* 1997; 75(1-2):41-46. The use of polyclonal antibody therapies for autoimmune diseases has been described by W. Cendrowski, *Boll Ist Sieroter Milan* 1997; 58(4):339-343; L.K. Kastrukoff et al., *Can J Neurol Sci* 1978; 5(2):175-178; J.E.
15 Walker et al., *J Neurol Sci* 1976; 29(2-4):303-309. The depletion of fat cells using antibody preparations has been described by L. De Clercq et al., *J Anim Sci* 1997; 75(7):1791-1797; J.T. Wright et al., *Obes Res* 1995; 3(3):265-272.

Regulatory elements in immunoglobulin genes have been described by Bradley et al. (1999), *Transcriptional enhancers and the evolution of the IgH locus*; Lauster, R. et 20 al., *Embo J* 12: 4615-23 (1993); Volgina et al., *J Immunol* 165:6400 (2000); Hole et al., *J Immunol* 146:4377 (1991).

Antibody diversification by gene conversion in chicken and rabbit has been described by Bucchini et al., *Nature* 326: 409-11 (1987); Knight et al., *Advances in Immunology* 56: 179-218 (1994); Langman et al., *Res Immunol* 144: 422-46 (1993). The 25 generation of mice expressing human-mouse chimeric antibodies has been described by Pluschke et al., *Journal of Immunological Methods* 215: 27-37 (1998). The generation of mice expressing human-mouse chimeric antibodies with mouse derived membrane and cytoplamic tails has been described by Zou et al., *Science* 262: 1271-1274 (1993); Zou et al. *Curr Biol* 4: 1099-1103. The generation of mice expressing human immunoglobulin 30 polypeptides has been described by Bruggemann et al. *Curr Opin Biotechnol* 8(4): 455-8 (1997); Lonberg et al. *Int Rev Immunol* 13(1):65-93 (1995); Neuberger et al., *Nature* 338:

350-2 (1989). Generation of transgenic mice using a BAC clone has been described by Yang et al., *Nat Biotechnol* 15: 859-65 (1997).

The generation of transgenic rabbits has been described by Fan, J. et al., *Pathol Int* 49: 583-94 (1999); Brem et al., *Mol Reprod Dev* 44: 56-62 (1996). Nuclear transfer cloning of rabbits has been described by Stice et al., *Biology of Reproduction* 39: 657-664 (1988). Rabbits with impaired immunoglobulin expression have been described by McCartney-Francis et al., *Mol Immunol* 24: 357-64 (1987); Allegrucci, et al., *Eur J Immunol* 21: 411-7 (1991).

The production of transgenic chicken has been described by Etches et al., 10 *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol* 12:415 (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the 15 germinal disc of the egg".

Agammaglobulinemic chicken have been described by Frommel et al., *J Immunol* 105(1): 1-6 (1970); Benedict et al., *Adv Exp Med Biol* 1977; 88(2): 197-205.

The cloning of animals from cells has been described by T. Wakayama et al., *Nature* 1998; 394:369-374; J.B. Cibelli et al., *Science* 280:1256-1258 (1998); J.B. Cibelli et al., *Nature Biotechnology* 1998; 16:642-646; A. E. Schnieke et al., *Science* 278: 2130-2133 (1997); K.H. Campbell et al., *Nature* 380: 64-66 (1996).

Production of antibodies from transgenic animals is described in U.S. Patent No. 5,814,318, No. 5,545,807 and No. 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for 25 introducing DNA into an embryo is described in U.S. Patent No. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent No. 5,453,357.

The mechanisms involved in the diversification of the antibody repertoire in pigs, sheep and cows are reviewed in Butler, J. E. (1998), "Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals", *Rev Sci Tech* 17:43. 30 Antibody diversification in sheep is described in Reynaud, C. A., C. Garcia, W. R. Hein,

and J. C. Weill (1995), "Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process", *Cell* 80:115; and Dufour, V., S. Malinge, and F. Nau. (1996), "The sheep Ig variable region repertoire consists of a single VH family", *J Immunol* 156:2163.

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Summary of the Invention

One embodiment of the present invention provides humanized antibodies (humanized immunoglobulins) having at least a portion of a human immunoglobulin polypeptide sequence.

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The humanized antibodies of the present invention are made from transgenic non-human animals genetically engineered to contain one or more humanized Ig loci.

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Preferably, the humanized antibodies of the present invention are prepared from transgenic non-human animals which generate antibody diversity primarily by gene conversion and hypermutation, e.g., rabbit, pigs, chicken, sheep, cow and horse. The antibodies can be made by immunizing transgenic animals with a desired antigen such as an infectious agent (e.g., bacteria or viruses) or parts or fragments thereof.

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Such humanized antibodies have reduced immunogenicity to primates, especially humans, as compared to non-humanized antibodies prepared from non-human animals. Therefore, the humanized antibodies of the present invention are appropriate for use in the therapeutic treatment of human subjects.

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Another embodiment of the present invention provides a preparation of humanized antibodies which can be monoclonal antibodies or polyclonal antibodies. Preferred antibody preparations of the present invention are polyclonal antibody preparations which, according to the present invention, have minimal immunogenicity to primates, especially humans.

30

A preferred preparation of polyclonal antibodies is composed of humanized immunoglobulin molecules having at least a heavy chain or light chain constant region polypeptide sequence encoded by a human constant region gene segment. More preferably, the variable domains of the heavy chains or light chains of the immunoglobulins molecules are also encoded by human gene segments.

In another embodiment, the present invention provides pharmaceutical compositions which include a preparation of humanized antibodies, and a pharmaceutically-acceptable carrier.

Another embodiment of the present invention provides novel sequences from 5 the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating the antibody diversity. In particular, the present invention provides novel nucleotide sequences downstream (3', 3-prime) of the genes coding for C λ in chickens, Cy and C κ in rabbits, Cy1,2,3 in cows and Cy1,2 in sheep, as well as novel sequences 5' of rabbit Cy.

10 In another embodiment, the present invention provides recombination vectors useful for replacing an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences are homologous to the flanking sequences of the target animal Ig gene segment.

15 Preferred recombination vectors are those useful for the replacement of the animal's Ig constant region. For example, recombination vectors useful for replacing the rabbit heavy chain constant region genes are provided. A preferred vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13, or a portion of SEQ ID NO: 12 or SEQ ID NO: 13, a human heavy chain constant region gene 20 segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of SEQ ID NO: 10. Another preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 51, which sequence is characterized as having a human Cy1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

25 Recombination vectors are also provided useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human C κ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain C κ 1 gene.

Other recombination vectors are provided which are useful for replacing the 30 chicken light chain constant region genes. A preferred vector contains a nucleotide

sequence as set forth in SEQ ID NO: 57 which is characterized as having a human C λ 2 linked to flanking sequences from the 5' and 3' flanking regions of the chicken light chain C λ gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

In still another embodiment, the present invention provides transgenic constructs or vectors containing at least one humanized Ig locus, i.e., an Ig locus from a non-human animal or a portion of an Ig locus from a non-human animal wherein the locus or the portion of a locus is genetically modified to contain at least one human Ig gene segment. Such humanized Ig locus has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulins.

One humanized Ig locus provided by the invention is a humanized heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in the humanized heavy chain locus are juxtaposed with respect to each other in an unrearranged, or partially or fully rearranged configuration. A preferred humanized heavy chain locus contains a human constant region gene segment, preferably, C α or C γ . A more preferred humanized locus contains multiple V gene segments and at least one human V gene segment, in addition to a human heavy chain constant region segment. The human V gene segment is placed downstream of the non-human V gene segments.

Another humanized Ig locus is a humanized light chain locus which includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed with respect to each other in an unrearranged or rearranged configuration. A preferred

humanized light chain locus contains a human constant region gene segment, preferably, C λ or C κ . More preferably, the humanized light chain locus further contains multiple V gene segments and at least one human V gene segment. The human V gene segment is placed downstream of the non-human V gene segments. Even more preferably, the 5 humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another embodiment of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus by isolating an Ig locus or a portion of an Ig locus from a non-human animal, and integrating the desired human Ig gene 10 segment(s) into the isolated animal Ig locus or the isolated portion of an Ig locus. The human Ig gene segment(s) are integrated into the isolated animal Ig locus or the isolated portion of an Ig locus by ligation or homologous recombination in such a way as to retain the capacity of the locus for undergoing effective gene rearrangement and gene 15 conversion in the non-human animal. Integration of a human Ig gene segment by homologous recombination can be accomplished by using the recombination vectors of the present invention.

In another embodiment, the present invention provides methods of making transgenic animals capable of producing humanized antibodies. The transgenic animals can be made by introducing a transgenic vector containing a humanized Ig locus, or a 20 recombination vector containing a human Ig gene segment, into a recipient cell or cells of an animal, and deriving an animal from the genetically modified recipient cell or cells.

Transgenic animals containing one or more humanized Ig loci, and cells derived from such transgenic animals (such as B cells from an immunized transgenic animal) are also provided. The transgenic animals of the present invention are capable of 25 gene rearranging and gene converting the transgenic humanized Ig loci to produce a diversified repertoire of humanized immunoglobulin molecules.

Brief Description of the Drawings

Figure 1. Cow Cy 3' flanking sequences. Primers are shown in shaded boxes. 30 The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3,

and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

5 **Figure 2.** Sheep Cy 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

Figure 3. A novel 3' flanking sequence (SEQ ID NO: 10) of the rabbit Cgamma gene.

10 **Figure 4.** A novel nucleotide sequence (SEQ ID NO: 11) 3' of the rabbit Ckappa 1 gene.

15 **Figure 5.** Novel nucleotide sequences (SEQ ID NO: 12 and SEQ ID NO: 13) 5' of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the M1 and M2 regions 3' of the Cgamma gene.

15 **Figure 7a.** DNA construct for the replacement of rabbit Ck with human Ck. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Ck1 gene. The upstream sequence (5'Ck) is 2.8 kb, the downstream sequence (3'Ck) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.

20 **Figure 7b.** DNA construct for the replacement of rabbit Cy with human Cy1. A 1.8 kb fragment containing a DNA sequence encoding human Cy1 is flanked by sequences from the rabbit Cy gene. The upstream sequence (5'Cy) is 1.9 kb, the downstream sequence (3'Cy) is 3.1 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

25 **Figure 8.** DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cy1 gene segment flanked by 50 nucleotides derived from the flanking regions of rabbit Cy gene. Flanking sequences derived from the flanking regions of rabbit Cy gene are underlined.

Figure 9. DNA fragment (SEQ ID NO: 52) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit VH1 and J genes are underlined.

5 **Figure 10.** DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin heavy chain C κ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa1 gene. Flanking sequences derived from the flanking regions of rabbit C κ gene are underlined.

10 **Figure 11.** DNA fragment (SEQ ID NO: 54) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit immunoglobulin V and J genes are underlined.

15 **Figure 12.** DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides (underlined) derived from the flanking sequences of chicken Clambda gene.

20 **Figure 13.** Modification of the chicken light chain locus using the ET system. A chicken genomic BAC clone with the full-length light chain locus was modified by homologous recombination. In a first step C λ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human C λ gene.

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from the flanking regions of chicken immunoglobulin V and J genes are underlined.

25 **Figure 15.** Modified chicken light chain locus.

Detailed Description of the Invention

One embodiment of the present invention provides humanized immunoglobulins (antibodies).

By "a humanized antibody" or "a humanized immunoglobulin" is meant an immunoglobulin molecule having at least a portion of a human immunoglobulin polypeptide sequence (or a polypeptide sequence encoded by a human Ig gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a 5 transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal.

The term "non-human animals" as used herein includes, but is not limited to, 10 rabbits, pigs, birds (e.g., chickens, turkeys, ducks, geese and the like), sheep, goats, cows and horses. Preferred non-human animals are those animals which rely primarily on gene conversion and/or somatic hypermutation to generate antibody diversity, e.g., rabbit, pigs, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow. Particularly preferred non-human animals are rabbit and chicken.

In animals such as human and mouse, there are multiple copies of V, D and J 15 gene segments on the heavy chain locus, and multiple copies of V and J gene segments on a light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, chicken, sheep, goat, and cow), however, gene rearrangement does not play a significant 20 role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, are used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene 25 segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion, a

process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene.

The term "Ig gene segment" as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals 5 and humans, and which are brought together in B cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments.

The term "human Ig gene segment" as used herein includes both naturally occurring sequences of a human Ig gene segment, degenerate forms of naturally occurring 10 sequences of a human Ig gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially identical to the polypeptide encoded by a naturally occurring sequence of a human Ig gene segment. By "substantially" is meant that the degree of amino acid sequence identity is at least about 85%-95%.

A preferred humanized immunoglobulin molecule of the present invention 15 contains at least a portion of a human heavy or light chain constant region polypeptide sequence. A more preferred immunoglobulin molecule contains at least a portion of a human heavy or light chain constant region polypeptide sequence, and at least a portion of a human variable domain polypeptide sequence.

In another embodiment of the present invention, a preparation of humanized 20 antibodies is provided.

By "a preparation of humanized antibodies" or "a humanized antibody preparation" is meant an isolated antibody product or a purified antibody product prepared from a transgenic non-human animal (e.g., serum, milk, or egg yolk of the animal) or from cells derived from a transgenic non-human animal (e.g., a B-cell or a hybridoma cell).

25 A humanized antibody preparation can be a preparation of polyclonal antibodies, which includes a repertoire of humanized immunoglobulin molecules. A humanized antibody preparation can also be a preparation of a monoclonal antibody.

Although the immunogenicity to humans of a humanized monoclonal antibody preparation is also reduced as compared to a non-humanized monoclonal antibody 30 preparation, humanized polyclonal antibody preparations are preferred embodiments of

the present invention. It has been recognized that humanized monoclonal antibodies still invoke some degree of an immune response (an anti-idiotype response) in primates (e.g., humans) when administered repeatedly in large quantities because of the unique and novel idiotype of the monoclonal antibody. The present inventors have uniquely recognized that 5 the overall immunogenicity of polyclonal antibodies is less dependent on an anti-idiotype response. For example, polyclonal antibodies made from non-human animals with only the constant region elements humanized (e.g., polyclonal antibodies having constant regions encoded by human gene segments, and having variable domains encoded by the 10 endogenous genes of the non-human animal), are substantially non-immunogenic to primates.

Without intending to be bound to any theory, the present inventors have proposed that the reduced immunogenicity of such a humanized polyclonal antibody preparation is due to the fact that the preparation contains a very large number of different 15 antibodies with many different idiotypes which are to a large extent defined by novel amino acid sequences in the complimentarity determining regions (CDR) of the heavy and light chain. Therefore, upon administration of such preparation into a primate such as a human, the administered amount of each individual immunoglobulin molecule in the preparation may be too low to solicit immune response against each immunoglobulin molecule. Thus, the humanized polyclonal antibody preparation which has many different 20 idiotypes and variable regions has minimal immunogenicity to a recipient, even if the antibodies in the polyclonal antibody preparation are all directed to the same antigen. To further reduce any potential residual immunogenicity, a humanized polyclonal antibody preparation may be prepared which is composed of immunoglobulin molecules having both the variable domains and the constant regions encoded by human Ig gene segments.

25 In a preferred embodiment, the present invention provides an antibody preparation which includes humanized immunoglobulin molecules having at least a portion of a human heavy or light chain constant region polypeptide sequence. More preferably, the humanized immunoglobulines in the antibody preparation of the present invention further contain at least a portion of a human variable domain polypeptide

sequence, in addition to at least a portion of a human constant region polypeptide sequence.

Preferred humanized antibody preparations of the present invention are composed of humanized antibodies made from transgenic non-human animals whose 5 antibody diversity is generated primarily by gene conversion, such as rabbit, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow; preferably, rabbit and chicken.

Once a transgenic non-human animal capable of producing diversified humanized immunoglobulin molecules is made (as further set forth below), humanized 10 immunoglobulins and humanized antibody preparations against an antigen can be readily obtained by immunizing the animal with the antigen. A variety of antigens can be used to immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead, fragments of the microorganisms, or antigenic molecules isolated from the 15 microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8, recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins, collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens 20 also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*, or culture supernatant from these bacteria cells. Other bacterial antigens which can be used in immunization include purified 25 lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and exotoxin from *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*.

Preferred antigens for the generation of antibodies against fungi include attenuated version of fungi or outer membrane proteins thereof, which fungi include, but are not limited to, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and 30 *Cryptococcus neoformans*.

Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited to respiratory syncytial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatitis B virus (HBV), cytomegalovirus (CMV), EBV, and 5 HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD20, CD22 and CD53 10 antigens (antibodies against which are useful for the treatment of B cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient 15 manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by 20 fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

For making a monoclonal antibody, spleen cells are isolated from the 25 immunized transgenic animal and used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From 30 Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion

Products Thereof"), WO 97/16537 ("Stable Chicken B-cell Line And Method of Use Thereof"), and EP 0 491 057 B1 ("Hybridoma Which Produces Avian Specific Immunoglobulin G"), the disclosures of which are incorporated herein by reference. In vitro production of monoclonal antibodies from cloned cDNA molecules has been 5 described by Andris-Widhopf et al., "Methods for the generation of chicken monoclonal antibody fragments by phage display", *J Immunol Methods* 242:159 (2000), and by Burton, D. R., "Phage display", *Immunotechnology* 1:87 (1995), the disclosures of which are incorporated herein by reference.

In a further embodiment of the present invention, purified monoclonal or 10 polyclonal antibodies are admixed with an appropriate pharmaceutical carrier suitable for administration in primates especially humans, to provide pharmaceutical compositions. Pharmaceutically acceptable carriers which can be employed in the present 15 pharmaceutical compositions can be any and all solvents, dispersion media, isotonic agents and the like. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the antibodies contained therein, its use in the pharmaceutical compositions of the present invention is appropriate. The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Examples of carriers include oils, water, saline solutions, alcohol, sugar, gel, lipids, liposomes, resins, porous matrices, binders, fillers, coatings, preservatives and the like, or combinations thereof 20

The present invention is further directed to novel nucleotide sequences and vectors, as well as the use of the sequences and vectors in making a transgenic non-human animal which produces humanized immunoglobulins.

In general, the genetic engineering of a non-human animal involves the 25 integration of one or more human Ig gene segments into the animal's genome to create one or more humanized Ig loci. It should be recognized that, depending upon the approach used in the genetic modification, a human Ig gene segment can be integrated at the endogenous Ig locus of the animal (as a result of targeted insertion, for example), or at a different locus of the animal. In other words, a humanized Ig locus can reside at the chromosomal location where the endogenous Ig locus of the animal ordinarily resides, or 30 at a chromosomal location other than where the endogenous Ig locus of the animal

ordinarily resides. Regardless of the chromosomal location, a humanized Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulin molecules. An Ig locus having the capacity to undergo gene 5 rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

In one embodiment, the present invention provides novel sequences useful for creating a humanized Ig locus and making transgenic animals capable of producing 10 humanized immunoglobulin molecules. In particular, the present invention provides sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating antibody diversity (e.g., rabbit, pigs, sheep, goat, cow, birds such as chicken, turkey, duck, goose, and the like).

15 The 5' and 3' flanking regions of the genes coding for the constant region are particularly important as these sequences contain untranslated regulatory elements (e.g., enhancers) critical for high Ig expression in the serum. The 3' flanking region of the genes coding for the constant region of the heavy chain also contain exons coding for the membranous and cytoplasmic tail of the membrane form of immunoglobulin (Volgina et 20 al. *J Immunol* 165:6400, 2000). It has been previously established that the membrane and cytoplasmic tail of the membrane form of antibodies are critical in achieving a high level of expression of the antibodies in mice sera (Zou et al., *Science* 262:1271, 1993). Thus, the identification of the flanking sequences permits the replacement of exons and 25 intervening introns of the Cy gene with the human equivalent, and the maintenance of the endogenous exons encoding the transmembrane and cytoplasmic tail regions as well as the endogenous non-coding enhancer sequences.

In one embodiment, the present invention provides 3' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences downstream (3', 3-prime) of the genes coding for rabbit Cy, cow Cy1,2,3, and 30 sheep Cy1,2 are provided. Especially preferred nucleotide sequences include SEQ ID NO:

10 (3' of rabbit C γ), SEQ ID NOS: 3-5 (3' of cow C γ 1,2,3), and SEQ ID NOS: 8-9 (3' of sheep C γ 1,2).

In another embodiment, the present invention provides 3' flanking sequences of light chain constant regions of non-human animals. More particularly, the present 5 invention provides nucleotide sequences downstream (3', 3-prime) of the genes coding for C κ in rabbits. Especially preferred nucleotide sequences include SEQ ID NO: 11 (3' of rabbit C κ).

In still another embodiment, the present invention provides 5' flanking sequences of heavy chain constant regions of non-human animals. More particularly, 10 nucleotide sequences upstream (5', 5-prime) of the rabbit C γ gene are provided. Especially preferred sequences include SEQ ID NO: 12 and SEQ ID NO: 13.

Another embodiment of the present invention provides 5' flanking sequences of light chain constant regions of non-human animals.

Portions of the above novel flanking sequences are provided by the present 15 invention. By "a portion" is meant a fragment of a flanking nucleotide sequence capable of mediating homologous recombination between the human Ig gene segment and the target animal Ig gene segment. Generally, a portion is at least about 200 base pairs, preferably, at least about 400 base pairs, for recombination in animal cells such as ES cells or fibroblasts, and at least about 40 base pairs, preferably at least about 50 base pairs, for 20 recombination in *E. coli*. Examples of portions of the above novel flanking sequences include SEQ ID NOS: 59-60, 61-62, 63-64, 65-66, 67-68 and 69-70 (represented by the underlined sequences in Figures 8-12 and 14, respectively).

In a further aspect, the present invention provides vectors useful for the replacement of an Ig gene segment of a non-human animal with the corresponding human 25 Ig gene segment. These vectors, also referred to herein as "recombination vectors", include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences have a degree of homology with the flanking sequences of the target animal Ig gene segment sufficient to mediate homologous recombination between the human gene and the animal gene segments. Generally, at least 30 about 200 bases should be identical between the flanking regions in a recombination

vector and the flanking regions of the target gene to achieve efficient homologous recombination in animal cells such as ES cells and fibroblasts; and at least about 40 bases should be identical to achieve efficient homologous recombination in *E. coli*.

Recombination vectors useful for replacing the animal's immunoglobulin 5 heavy chain constant region genes are provided, which contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target animal heavy chain constant region gene, a human heavy chain constant region gene (e.g., human C γ 1), and a nucleotide sequence homologous to the 3' flanking region of the target animal heavy chain constant region gene.

Preferred recombination vectors are provided for the replacement of the rabbit 10 heavy chain constant region genes. One such vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13 or a portion thereof, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another such vector contains SEQ ID NO: 51 15 (Figure 8) which is characterized as having a human C γ 1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided which are useful for replacing the animal's immunoglobulin light chain constant region genes. Such vectors contain from 5' 20 to 3', a nucleotide sequence homologous to the 5' flanking region of the target light chain constant region gene, a human light chain constant region gene (e.g., human C κ or C λ), and a nucleotide sequence homologous to the 3' flanking region of the target light chain constant region gene.

Preferred vectors include those useful for replacing the rabbit light chain 25 constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human C κ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain C κ 1 gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing 30 a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A

recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

The recombination vectors of the present invention can include additional sequences that facilitate the selection of cells which have undergone a successful 5 recombination event. For example, marker genes coding for resistance to neomycin, bleomycin, puromycin and the like can be included in the recombination vectors to facilitate the selection of cells which have undergone a successful recombination event.

In a further aspect of the present invention, transgenic constructs or vectors carrying one or more humanized Ig loci are provided.

10 In one embodiment, the present invention provides transgenic constructs containing a humanized Ig heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in such humanized heavy chain locus are juxtaposed 15 with respect to each other in an unarranged configuration (or "the germline configuration"), or in a partially or fully rearranged configuration. The humanized heavy chain locus has the capacity to undergo gene rearrangement (if the gene segments are not fully rearranged) and gene conversion in the non-human animal thereby producing a diversified repertoire of heavy chains having human polypeptide sequences, or 20 "humanized heavy chains".

In a preferred embodiment, the humanized heavy chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, C α or C γ (including any of the C γ subclasses 1, 2, 3 and 4).

25 In another more preferred embodiment, the humanized heavy chain locus of the transgene contains a humanized V-region and a humanized C-region, i.e., a V-region having at least one human VH gene segment and a C-region having at least one human C gene segment (e.g., human C α or C γ).

30 Preferably, the humanized V-region includes at least about 10-100 heavy chain V (or "VH") gene segments, at least one of which is a human VH gene segment. In accordance with the present invention, the human VH gene segment included in the

transgene shares at least about 75% to about 85% homology to the VH gene segments of the host animal, particularly those animal VH gene segments included in the upstream region of the transgene. As described above, a human VH segment encompasses naturally occurring sequences of a human VH gene segment, degenerate forms of naturally occurring sequences of a human VH gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human heavy chain V domain polypeptide.

5

Preferably, the human VH gene segment(s) is placed downstream of the non-human VH segments in the transgene locus. Preferably, the non-human VH gene segments in the transgene are the VH gene segments from the 3' VH-region in the Ig locus of the host animal, including the 3' proximal VH1.

10

In another embodiment, the present invention provides transgenic constructs containing a humanized light chain locus capable of undergoing gene rearrangement and gene conversion in the host animal thereby producing a diversified repertoire of light chains having human polypeptide sequences, or "humanized light chains".

15

The humanized light locus includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed in an unrearranged configuration (or "the germline configuration"), or fully rearranged configuration.

20

In a preferred embodiment, the humanized light chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, C λ or C κ .

In another preferred embodiment, the humanized light chain locus of the transgene contains a humanized V-region and a humanized C-region, e.g., a V-region having at least one human VL gene and/or at least one rearranged human VJ segment, and a C-region having at least one human C gene segment (e.g., human C λ or C κ).

25

Preferably, the humanized V-region includes at least about 10-100 light chain V (or "VL") gene segments, at least one of which is a human VL gene segment. The 30 human VL gene segment included in the transgene shares at least about 75% to about 85%

homology to the VL gene segments of the host animal, particularly those animal VL gene segments included in the upstream region of the transgene. Consistently, a human VL segment encompasses naturally occurring sequences of a human VL gene segment, degenerate forms of naturally occurring sequences of a human VL gene segment, as well 5 as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human light chain V domain polypeptide.

Preferably, the human VL gene segment(s) is placed downstream of the non-human VL segments in the transgene locus. The non-human VL gene segments in the transgene construct are selected from the VL gene segments in the 3'VL-region in the 10 light chain locus of the host animal, including the 3' proximal VL1.

In still another preferred embodiment, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another aspect of the present invention is directed to methods of making a 15 transgenic vector containing a humanized Ig locus. Such methods involve isolating an Ig locus or a portion thereof from a non-human animal, and inserting the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an animal Ig locus. The human Ig gene segment(s) are inserted into the isolated animal Ig locus or a portion thereof by ligation or homologous recombination in such a way as to retain the 20 capacity of the locus of undergoing effective gene rearrangement and gene conversion in the non-human animal.

Preferably, DNA fragments containing an Ig locus to be humanized are 25 isolated from animals which generate antibody diversity by gene conversion, e.g., rabbit and chicken. Such large DNA fragments can be isolated by screening a library of plasmids, cosmids, YACs or BACs, and the like, prepared from the genomic DNA of the non-human animal. An entire animal C-region can be contained in one plasmid or cosmid clone which is subsequently subjected to humanization. YAC clones can carry DNA fragments of up to 2 megabases, thus an entire animal heavy chain locus or a large portion thereof can be isolated in one YAC clone, or reconstructed to be contained in one YAC 30 clone. BAC clones are capable of carrying DNA fragments of smaller sizes (about 150-

250 kb). However, multiple BAC clones containing overlapping fragments of an Ig locus can be separately humanized and subsequently injected together into an animal recipient cell, wherein the overlapping fragments recombine in the recipient animal cell to generate a continuous Ig locus.

5 Human Ig gene segments can be integrated into the Ig locus on a vector (e.g., a BAC clone) by a variety of methods, including ligation of DNA fragments, or insertion of DNA fragments by homologous recombination. Integration of the human Ig gene segments is done in such a way that the human Ig gene segment is operably linked to the host animal sequence in the transgene to produce a functional humanized Ig locus, i.e., an 10 Ig locus capable of gene rearrangement and gene conversion which lead to the production of a diversified repertoire of humanized antibodies.

Preferably, human Ig gene segments are integrated into the Ig locus by homologous recombination. Homologous recombination can be performed in bacteria, yeast and other cells with a high frequency of homologous recombination events. For 15 example, a yeast cell is transformed with a YAC containing an animal's Ig locus or a large portion thereof. Subsequently, such yeast cell is further transformed with a recombination vector as described hereinabove, which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector are homologous to those flanking sequences of the animal Ig gene 20 segment on the YAC. As a result of a homologous recombination, the animal Ig gene segment on the YAC is replaced with the human Ig gene segment. Alternatively, a bacterial cell such as *E. coli* is transformed with a BAC containing an animal's Ig locus or a large portion thereof. Such bacterial cell is further transformed with a recombination vector which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' 25 flanking sequence. The 5' and the 3' flanking sequences in the recombination vector mediate homologous recombination and exchange between the human Ig gene segment on the recombination vector and the animal Ig gene segment on the BAC. Humanized YACs and BACs can be readily isolated from the cells and used in making transgenic animals.

In a further aspect of the present invention, methods of making transgenic 30 animals capable of producing humanized immunoglobulins are provided.

According to the present invention, a transgenic animal capable of making humanized immunoglobulins are made by introducing into a recipient cell or cells of an animal one or more of the transgenic vectors described herein above which carry a humanized Ig locus, and deriving an animal from the genetically modified recipient cell or 5 cells.

Preferably, the recipient cells are from non-human animals which generate antibody diversity by gene conversion and hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3' proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V 10 gene segment into the Ig locus on the transgene vector, either by replacing the 3' proximal V gene segment of the animal or by being placed in close proximity of the 3' proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

15 The transgenic vectors containing a humanized Ig locus is introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a humanized Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For 20 example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem 25 cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the humanized Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a preferred embodiment, a transgene containing a humanized Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential 5 expression of immunoglobulin molecules from the humanized transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as Agammaglobinemic chicken strain. Alternatively, transgenic animals with humanized immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired 10 endogenous Ig locus and a humanized transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be 15 selected by standard methods. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Enucleation of oocytes and nuclear transfer can also be 20 performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369.) The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

25 Further to the present invention, a transgenic animal capable of producing humanized immunoglobulins can also be made by introducing into a recipient cell or cells, one or more of the recombination vectors described herein above, which carry a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, selecting cells in which the endogenous Ig

gene segment is replaced by the human Ig gene segment by homologous recombination, and deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated 5 somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure 10 for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one 15 or more, humanized Ig loci in the genome, from which a functional repertoire of humanized antibodies is produced.

In a preferred embodiment, the present invention provides transgenic rabbits having one or more humanized Ig loci in the genome. The transgenic rabbits of the present invention are capable of rearranging and gene converting the humanized Ig loci, 20 and expressing a functional repertoire of humanized antibodies.

In another preferred embodiment, the present invention provides transgenic chickens having one or more humanized Ig loci in the genome. The transgenic chickens of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

25 Cells derived from the transgenic animals of the present invention, such as B cells or cell lines established from a transgenic animal immunized against an antigen, are also part of the present invention.

In a further aspect of the present invention, methods are provided for treating a disease in a primate, in particular, a human subject, by administering a purified humanized

antibody composition, preferably, a humanized polyclonal antibody composition, desirable for treating such disease.

The humanized polyclonal antibody compositions used for administration are generally characterized by containing a polyclonal antibody population, having 5 immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody composition may contain immunoglobulins of various isotypes. Alternatively, the antibody composition may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody composition consists of unmodified 10 immunoglobulins, i.e., humanized antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody compositions generally are administered into the vascular 15 system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody composition is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be accepted by the patient. Administration of the effective dosage may occur in a single 20 infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody compositions are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

25 The antibody compositions can be used to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably are 30 also capable of stimulating an immune response.

Administration of an antibody composition against an infectious agent as a monotherapy or in combination with chemotherapy results in elimination of infectious particles. A single administration of antibodies decreases the number of infectious particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly, 5 antibody therapy in patients with a malignant disease employed as a monotherapy or in combination with chemotherapy reduces the number of malignant cells generally 10 to 100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of time to assure the complete elimination of infectious particles, malignant cells, etc. In some instances, therapy with antibody preparations will be continued for extended periods 10 of time in the absence of detectable amounts of infectious particles or undesirable cells. Similarly, the use of antibody therapy for the modulation of immune responses may consist of single or multiple administrations of therapeutic antibodies. Therapy may be continued for extended periods of time in the absence of any disease symptoms.

The subject treatment may be employed in conjunction with chemotherapy at 15 dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease patients or transplant recipients, antibody therapy may be employed in conjunction with immunosuppressive therapy at dosages sufficient to inhibit immune reactions.

The invention is further illustrated, but by no means limited, by the following examples.

20

Example 1

25 **Novel Sequences 3'prime of the Cy Gene from Cows, Sheep and Rabbits**

Genomic DNA was isolated from blood of a Simmental cow using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the cow Cy gene (i.e., the cow Cy gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA 30 as template and the following primers:

5' primer: 5'cgcaagcttCCTACACGTGTGGTGATG3' (SEQ ID NO: 1);

3' primer: 5'cgcaagcttAAGATGGWGATGGTSGTCCA3' (SEQ ID NO: 2)(Universal degenerate code: W=(A/T) S=(G/C)).

The upper-case portion of the 5' primer was from exon 3 of Cy, and the lower-case portion represented a terminal HindIII restriction site. The upper-case portion of the 3' primer was a degenerate sequence designed according to the published sequences from the human M1 exon and the mouse M1 exon, and the lower-case portion represented a terminal HindIII restriction site. A 1.3kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with HindIII, and cloned into a Bluescript cloning vector. The resulting clones fell into three populations, which differ from one another in the pattern of the restriction fragments obtained with BamHI, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 1 (SEQ ID NOS: 3-5).

10 Genomic DNA was isolated from blood of a Merino sheep using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the sheep Cy gene (i.e., the sheep 15 Cy gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgccgatccCCTACGCGTGTGGTGATG3' (SEQ ID NO: 6)

3' primer: 5'cgccgatccACCGAGGAGAAGATCCACTT3' (SEQ ID NO: 7)

17 The upper-case portion of the 5' primer was from exon 3 of Cy, and the lower-case 20 portion represented a terminal BamHI restriction site. The upper-case portion of the 3' primer was designed according to the published sequences from the human M2 exon and the mouse M2 exon, and the lower-case portion represented a terminal BamHI restriction site. A 2.9kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with BamHI, and cloned into a 25 Bluescript cloning vector. The resulting clones fell into two populations, which differ from each other in the pattern of the restriction fragments obtained with HindIII, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 2 (SEQ ID NOS: 8-9).

27 A 10kb EcoRI fragment containing the Cy gene and its flanking sequences 30 from A2 allotype rabbit was subcloned from a genomic cosmid clone (cos 8.3 from

Knight et al., *J Immunol* (1985) 1245-50, "Organization and polymorphism of rabbit immunoglobulin heavy chain genes"). The nucleotide sequences 5' and 3' of Cy were determined using standard methods and are set forth in Figure 3 and 5, SEQ ID NO: 10, 12, 13, respectively.

5 Sequences 3' of rabbit Ckappa1 were determined from an EcoRI/BamHI subclone from VJk2Ck In pSV2neo. The nucleotide sequence is set forth in Figure 4, SEQ ID NO: 11.

10 The amino acid sequences encoded by the M1 and M2 exons from cow, sheep and rabbit were deduced from the above 3' flanking sequence. These amino acid sequences were aligned with the published M1 and M2 sequences from camel, human and mouse, as shown in Figure 6.

Example 2

15 **A Vector for Replacing the Rabbit Endogenous Cy Gene Segment with the Human Cy1 Segment**

Genomic DNA is isolated from rabbit fetal fibroblasts of an a2-homozygous rabbit. The DNA sequence upstream of rabbit Cy (i.e., the 5' flanking sequence of rabbit Cy) is amplified by PCR using the following primers:

20 5' taattatgcggccgcCTTCAGCGTGAACCACGCCCTC 3' (SEQ ID NO: 39)

with a 5' NotI site and

5' GTCGACGCCCTCGATGCACCTCCAGAG 3' (SEQ ID NO: 40).

The DNA sequence downstream of rabbit Cy (i.e., the 3' flanking sequence of rabbit Cy) is amplified with the following primers:

25 5' ggtaccCTCTCCCTCCCCACGCCGCAGC 3' (SEQ ID NO: 41) with a 5'

KpnI site and

5' atatctcagaACTGGCTGCCCTGCTGTAGTACACGG 3' (SEQ ID NO: 42)

with a 5' XhoI site.

Human genomic DNA is isolated from human peripheral blood lymphocytes.

30 The DNA fragment encoding human Cy1 is amplified using the following primers:

5' GTCGACACTGGACGCTGAACCTCGCGG 3' (SEQ ID NO: 43) and

5' GGTACCGGGGGCTGCCGGCGTCGCAC 3' (SEQ ID NO: 44).

The fragments are digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette is inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in 5 Figure 7a.

Example 3

A Vector for Replacing the Rabbit Endogenous C_K Gene Segment with the Human C_K Segment

10

Genomic DNA was isolated from rabbit fetal fibroblasts of a b5-homozygous rabbit. The DNA sequence upstream of rabbit C_K1 (i.e., the 5' flanking sequence of rabbit C_K1) was amplified by PCR using the following primers:

15 5' gcggccgcTGGCGAGGAGACCAAGCTGGAGATCAAACG 3' (SEQ ID NO: 45) with a 5' NotI site

5' GTCGACGCAGCCAAAGCTGGCAATGGGGCAGCG 3' (SEQ ID NO: 46).

The DNA sequence downstream of rabbit C_K1 (i.e., the 5' flanking sequence of rabbit C_K1) was amplified with the following primers:

20 5' atatggtaccGCGAGACGCCCTGCCAGGGCACCGCC 3' (SEQ ID NO: 47) with a 5' KpnI site

5' GGATCCCGAGCTTATGGGCAGGGTGGGG 3' (SEQ ID NO: 48).

Human genomic DNA was isolated from human peripheral blood lymphocytes. The DNA fragment encoding human C_K was amplified using the following 25 primers:

5' ATATGTCGACCTGGATAAGCATGCTGTTCTGTCTGTCCC 3' (SEQ ID NO: 49)

5' CTAGGTACCAGCAGGTGGGGCACTTCTCCC 3' (SEQ ID NO: 50).

The fragments were digested with restriction enzymes and cloned into a Bluescript vector. 30 Subsequently, a lox neo-cassette was inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7b.

Example 4**Replacement of the Endogenous Cy and Ck Gene Segments in Rabbit Fetal Fibroblasts with the Corresponding Human Gene Segments**

5

Rabbit fetal fibroblast cells are prepared by standard methods. After one passage, fibroblasts are transfected with 5 μ g of the NotI-linearized targeting vector as shown in Figure 5a for Cy or Figure 51b for Ck, and are seeded in 96-well plates (2×10^3 cells/well). After a positive selection with 600 μ g/ml G418 and a negative selection with 10 200nM FIAU, resistant colonies are replica-plated to two 96-well plates for DNA analysis and cryopreservation, respectively. PCR and/or Southern blot analysis is performed to identify cells with the human Cy1 gene segment integrated in the genome. The cells having the integrated human Cy1 gene are used in rabbit cloning as described in Example 10 5.

15

Example 5**Cloning of Rabbits**

Mature Dutch Belton rabbits are superovulated by subcutaneous injection of 20 follicle stimulating hormone (FSH) every 12 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by oviductal flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation. Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St. 25 Louis, MO) dye under ultraviolet light. Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15 us (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6 uM) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty 30 Media, Lavalett, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture

medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 2-48 hours at 37.8° C and 5%CO₂ in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

5

Example 6

10 Construction of a DNA Fragment Containing a Portion of a Rabbit Heavy Chain Locus with a Human Cy1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit heavy chain Cy gene from an a2-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 51) is generated by PCR using overlapping oligonucleotides 15 wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cy gene, the human Cy1 gene, and a sequence derived from the 3' flanking region of the rabbit Cy gene (Figure 8).

A genomic BAC library derived from an a2-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cy. A BAC clone 20 containing rabbit heavy chain gene segments is identified. The rabbit Cy gene on this BAC clone is replaced with the human Cy1 gene by homologous recombination in *E.coli* using the DNA fragment of SEQ ID NO: 51 and the pET system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cy gene segment is replaced with a marker gene; then the marker gene is replaced the human Cy1 gene 25 segment.

The modified BAC clone containing rabbit heavy chain genes and the inserted human Cy1 gene is further modified by replacing the 3'proximal VH1 segment with a synthetic VH gene segment (Figure 9). This synthetic VH gene segment (SEQ ID NO: 52) is made using overlapping oligonucleotides and includes a 5' flanking sequence, a 3' flanking sequence, and a sequence coding for a polypeptide nearly identical to the human 30 immunoglobulin heavy chain variable domain polypeptide sequence described by Huang

and Stollar (*J. Immunol.* 151: 5290-5300, 1993). The coding sequence of the synthetic VH gene segment is designed based on the published sequence of a rabbit VH1 gene (a2, Knight and Becker, *Cell* 60:963-970, 1990) and is more than 80% identical to rabbit VH gene segments. The 5' and the 3' flanking sequences in the synthetic VH segment are 5 derived from the upstream and downstream regions of the a2-allotype rabbit VH1 gene. The synthetic VH gene of SEQ ID NO: 52 is used to replace the rabbit VH1 gene on the BAC clone by homologous recombination using the pET or the red β system. The modified BAC clone is amplified and purified using standard procedures.

10

Example 7

Construction of a DNA Fragment Containing a Portion of a Rabbit Light Chain Locus with a Human C κ 1 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain Polypeptide Sequence

15

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit light chain C κ 1 gene from a b5-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 53) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit C κ 1 gene, the human C κ 1 gene, and a sequence derived from the 3' flanking region of the rabbit C κ 1 gene (Figure 10).

A genomic BAC library derived from a b5-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit C κ 1. A BAC clone containing rabbit light chain gene segments is identified. The rabbit C κ 1 gene on this 25 BAC clone is replaced with the human C κ 1 gene on the DNA fragment of SEQ ID NO: 53 by homologous recombination in *E. coli* using the pET or the red β system. This replacement is accomplished by two consecutive recombination steps: first the rabbit C κ 1 gene segment is replaced with a marker gene; then the marker gene is replaced the human C κ 1 gene segment.

30 The modified BAC clone containing rabbit light chain genes and the inserted human C κ 1 gene is further modified by inserting a rearranged VJ DNA fragment into the

J region of the rabbit light chain locus. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Pritsch et al. (*Blood* 82(10):3103-3112, 1993) and Lautner-Rieske et al. (*Eur. J. Immunol.* 22 (4), 1023-1029, 1992)) (Figure 7). The nucleotide sequence of the rearranged VJ fragment is designed to 5 maximize the sequence homology at the nucleotide level to the rabbit V κ appa sequence published by Lieberman et al. (*J. Immunol.* 133 (5), 2753-2756, 1984). This rearranged VJ DNA sequence is more than 80% identical with known rabbit V κ genes. Using overlapping oligonucleotides in PCR, the rearranged VJ DNA fragment is linked to a 5' and a 3' flanking sequence, resulting the DNA fragment of SEQ ID NO: 54 (Figure 11).
10 The 5'flanking sequence is derived from 5' of a rabbit V κ , the 3'flanking sequence is derived from 3' of rabbit J2. The DNA fragment of SEQ ID NO: 54 is subsequently inserted into the rabbit light chain locus by homologous recombination in *E. coli* using the pET or the red β system. The insertion is performed in such a way that the rabbit light chain region containing the rabbit V κ 1 gene segment, the rabbit J1 and J2 segments, and 15 the sequences in between, is replaced with the rearranged VJ DNA fragment. Again, this insertion is accomplished by replacement of the rabbit V to J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified BAC clone is amplified and purified using standard procedures.

20

Example 8

Transgenic Rabbits Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgene

25 Transgenic rabbits are generated as described by Fan et al. (*Pathol. Int.* 49: 583-594, 1999). Briefly, female rabbits are superovulated using standard methods and mated with male rabbits. Pronuclear-stage zygotes are collected from oviduct and placed in an appropriate medium such as Dulbecco's phosphate buffered saline supplemented with 20% fetal bovine serum. The exogenous DNA (e.g., the humanized BAC clone from 30 Example 4 and/or 5 which has been linearized prior to injection) is microinjected into the male pronucleus with the aid of a pair of manipulators. Morphological surviving zygotes

are transferred to the oviducts of pseudopregnant rabbits. Pseudopregnancy is induced by the injection of human chorionic gonadotrophin (hCG). Between about 0.1-1% of the injected zygotes develop into live transgenic rabbits. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene.

5 cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) of a transgenic rabbit. Primers specific for the human transgene (human CH gene segment or the synthetic humanized VH gene segment) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is
10 transcribed in the animal. Amplified products are sequenced and the presence of donor sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

15 The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic founder rabbits is determined using an ELISA assay.

20

Example 9

Production of Humanized Antibodies From Transgenic Rabbits with the Genetic Background of the Alicia and/or Basilea Rabbit Strain

25 The Alicia strain lacks the VH1 gene segment and therefore has an impaired Ig heavy chain expression. Transgenic founder rabbits capable of expressing humanized heavy chain molecules in the genetic background of the Alicia rabbit strain are generated, e.g., by using fetal fibroblasts established from Alicia rabbits in Examples 4-5 above, or by using zygotes from female Alicia rabbits mated with male Alicia rabbits in Example 8
30 above. Transgenic animals are also obtained which are homozygous for the Alicia Ig phenotype and are also homozygous for a humanized heavy chain transgene. Serum is tested in ELISA for the presence of humanized heavy chain (e.g., a human heavy chain

constant region). The concentration of antibodies with humanized Ig heavy chains in these homozygous Alicia animals is substantially higher, e.g., about 10 to 100 fold higher, than that produced from a transgene integrated in the genome of wild type (non-Alicia) rabbits.

5 The Basilea strain does not express $\kappa 1$ light chain and in its place exclusively express the $\kappa 2$ and λ light chains. Transgenic founder rabbits capable of expressing humanized light chain molecules in the genetic background of the Basilea rabbit strain are generated, e.g., by using fetal fibroblasts established from Basilea rabbits in Examples 4-5 above, or by using zygotes from female Basilea rabbits mated with male Basilea rabbits in 10 Example 8 above. Transgenic animals are obtained which are homozygous for the Basilea light chain phenotype, and are also homozygous for a humanized light chain transgene. Serum is tested in ELISA for the presence of the humanized light chain. The concentration of the humanized light chain in the homozygous Basilea animals is substantially higher, about 10-100 fold higher, than the concentration of a humanized light 15 chain in a transgenic rabbit with the wild type (non-Basilea) genetic background. Transgenic founder rabbits are mated with each other to generate transgenic rabbits with the following traits: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, (3) homozygous for the Alicia heavy chain locus, and (4) homozygous for the Basilea light chain locus.

20

Example 10

Construction of a DNA Fragment Containing a Modified Chicken Light Chain Locus Having a Human Clambda2 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain

25

A genomic BAC library derived from a jungle fowl chicken was screened with radiolabeled probes specific for chicken light chain Clambda and chicken Vpsi25 (the V gene segment at the very 5' end of the light chain locus). A BAC clone containing the entire lambda light chain locus was identified. The chicken C λ gene on this BAC clone is 30 replaced with the human C λ 2 gene by homologous recombination in *E.coli* using the pET system (Zhang et al., *Nat. Biotechnol.* 18(12):1314-7, 2000) as follows.

A first DNA fragment containing a kanamycin selection cassette was generated by PCR using primers specific for Tn5 gene. The 5' primer (5'catacacagccatacatacgcgtgtggccgctgcctctcttgcaggTATGGACAGCAAGCGAACCG 3', SEQ ID NO: 55) was designed to include 50 bp at the 5' end (lower case), derived 5 from the 5' flanking region of the chicken light chain C λ gene. The 3' primer (5'atcagggtgacccttacgttacactccgtcaccaaggagtggagggacTCAGAAGAACTCGTCAAGA AG3', SEQ ID NO: 56) was designed to include about 50 bp at the end (lower case), derived from the 3' flanking region of the chicken light chain C λ gene.

10 A second DNA fragment (SEQ ID NO: 57) was synthesized using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the chicken light chain Clambda gene, the human Clambda2 gene, and a sequence derived from the 3' flanking region of the chicken Clambda gene (Figure 12).

15 *E. coli* cells of the chicken light chain BAC clone were transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid were then transformed with the first DNA fragment above and selected afterwards in media containing kanamycin. Clones resistant to kanamycin were identified, and the replacement of the chicken C λ segment by the kanamycin selection cassette via homologous recombination was 20 confirmed by restriction enzyme digest.

25 In the second homologous recombination step, cells positive for the kanamycin selection cassette were transformed with the second DNA fragment above. Transformed cells were screened for the loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human C λ 2 gene. The exchange was confirmed by restriction enzyme digest and/or sequence analysis.

The ET cloning procedure is summarized in Figure 13.

30 The BAC clone containing the chicken light chain locus and the inserted human Clambda2 gene segment was further modified by inserting a rearranged VJ DNA fragment. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Kametani et al. (*J. Biochem.* 93 (2), 421-429, 1983) as

IG LAMBDA CHAIN V-I REGION NIG-64 (P01702) (Figure 14). The nucleotide sequence of the rearranged VJ fragment was so designed as to maximize the sequence homology at the nucleotide level to the chicken Vlambda1 sequence published by McCormack et al. (*Cell* 56, 785-791, 1989). This rearranged VJ DNA sequence is more than 80% identical with known chicken light chain V genes. The rearranged VJ DNA fragment was linked to a 5' flanking sequence and a 3' flanking sequence, resulting in the DNA fragment of SEQ ID NO: 58 (Figure 14). The 5' flanking sequence was derived from 5' of chicken Vlambda1, and the 3' flanking sequence was derived from 3' of chicken J. The DNA fragment of SEQ ID NO: 58 was subsequently inserted into the chicken light chain locus in *E. coli* using the pET system as shown in Figure 15. The insertion was performed in such a way that the region on the chicken light chain locus from the 5' end of the chicken Vlambda1 gene segment to the 3' end of the chicken J region was replaced with the rearranged, synthetic VJ DNA fragment. Again, this insertion was accomplished by the replacement of the chicken V-J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified region of the chicken light chain locus is shown in Figure 15. The modified BAC clone was amplified and purified using standard procedures.

Example 11

20 **Construction of a DNA Fragment Containing a Portion of a Chicken Heavy Chain Locus With a Human Cy1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence**

25 A jungle fowl chicken genomic BAC library was generated by standard procedures and screened with probes specific for chicken Cy. A BAC clone containing chicken heavy chain gene segments is identified. The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the heavy chain Cy gene are sequenced. The chicken Cy gene on this BAC clone is replaced with the human Cy1 gene by homologous recombination in *E. coli* using the pET system as follows.

30 A first DNA fragment containing a kanamycin selection cassette is generated by PCR using primers specific for Tn5 gene. The 5' and 3' primers are designed to

include about 50 bp at the end, derived from the 5' and 3' flanking regions of the chicken heavy chain Cy gene.

A second DNA fragment is generated by PCR using overlapping oligonucleotides wherein this second DNA fragment contains from 5' to 3', a sequence of 5 about 50 bp derived from the 5' flanking region of the chicken Cy gene, the human Cy1 gene, and a sequence of about 50 bp derived from the 3' flanking region of the chicken Cy gene.

10 *E. coli* cells of the chicken CY BAC clone are transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid are further transformed with the first DNA fragment and selected in media containing kanamycin. Clones resistant to kanamycin are identified, and the replacement of the chicken CY segment by the kanamycin selection cassette via homologous recombination is confirmed by restriction enzyme digest.

15 In the second homologous recombination step, cells positive for the kanamycin selection cassette are now transformed with the second DNA fragment described above. Transformed cells are screened for loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cy1 gene. The exchange is confirmed by restriction enzyme digest and/or sequence analysis.

20 The BAC clone containing the inserted human Cy1 gene is further modified by replacing the 3' proximal VH1 segment (i.e., the 3' proximal VH1 gene in the V region) with a synthetic VH gene segment. This synthetic VH gene segment is designed based on the published sequence of a chicken VH1 gene (Arakawa et al., EMBO J 15(10): 2540-2546, 1996). The synthetic gene segment is more than 80% identical to chicken VH gene 25 segments and encodes an amino acid sequence that is identical to the amino acid sequence of a human immunoglobulin heavy chain variable domain polypeptide described by Matthysse and Rabbitss (in Steinberg CM and Lefkovits I, (eds). *The Immune System*: 132-138, S. Karger, NY 1981). This synthetic VH segment including 5' and 3' flanking sequences is synthesized by PCR using overlapping oligonucleotides. The 5' and the 3' 30 flanking sequences are derived from the upstream and downstream regions of chicken

VH1 gene. This synthetic VH segment is used to replace the chicken VH1 gene on the BAC clone by homologous recombination using the pET system. The modified BAC clone is amplified and purified using standard procedures.

5

Example 12

Transgenic Chicken Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgenes

The production of transgenic chicken is carried out using techniques as described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol* 12:415 (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Briefly, the modified BAC clones are linearized and mixed with a transfection reagent to promote uptake of DNA into cells. The formulations are injected into a multicell stage chicken embryo in close proximity to the germinal disc. The window in the egg shell is closed and the eggs are incubated. After hatching chimeric chickens are identified by PCR and Southern blot analysis using transgene specific sequences. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene. Heavy and light chain transgenic animals are bred with each other to generate transgenic chickens expressing antibodies having humanized heavy and light chains.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) from transgenic chickens. Primers specific for the human transgene (e.g., human CH gene segments and/or the synthetic humanized VH gene segments) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor

sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic chickens is determined using an 5 ELISA assay.

Example 13

**Production of Functional Humanized Antibodies
in Transgenic Chicken with the Agammaglobulinemic Phenotype**

10

Transgenic chickens with the following traits are produced: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, and (3) homozygous for the agammaglobulinemic phenotype. These animals produce antibodies into the blood and eggs, and antibodies can be purified from either 15 source. In general, antibody concentrations in the eggs are about 5% to 50% of antibodies concentration in the blood. Animals that contain humanized antibodies at high levels in eggs can be selected and bred to produce offspring. Alternatively, transgenic animals can be generated that specifically secrete humanized antibodies into their eggs.

20

Example 14

**Generation Of Transgenic Chickens
Expressing Humanized Immunoglobulin**

25

Chicken embryonic stem cells are isolated and cultured as described by Pain et al. (*Development* 122, 2339-2348; 1996). Chicken embryos are obtained from eggs immediately after they are laid. The entire blastoderm is removed by gentle aspiration, embryos are slowly dissociated mechanically and cells are seeded in ESA complete 30 medium on inactivated STO feeder cells. ESA medium is composed of MEM medium containing 10% FCS, 2% chicken serum, 1% bovine serum albumin, 10 ng/ml ovalbumin, 1 mM sodium pyruvate, 1% non-essential amino acids, 1 μ M of each nucleotide

adenosine, guanosine, cytidine, uridine, thymidine, 0.16 mM β -mercaptoethanol, ESA complete medium is supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11. Cell cultures are incubated wt 37°C in 7.5 CO₂ and 90% humidity. After 48 hours fresh blastodermal cells are added to 5 the culture in half of the original volume of ESA complete medium. After an additional incubation for three days, the culture medium is partially (50%) replaced with fresh ESA complete medium, and totally every day thereafter. For cell harvesting, cultures are washed with PBS and incubated in a pronase solution (0.025% w/v). Dissociated cells are transfected with various linearized transgenic constructs containing a humanized Ig locus. 10 Transfected cells are incubated with STO feeder cells (as described above) in the presence of selective antibiotics. Cells are transferred onto fresh feeder cells twice per week. Antibiotic resistant cells are isolated and the integration of a humanized Ig gene fragments at a random site or at the corresponding chicken immunoglobulin gene loci is confirmed by PCR. 15 Subsequently, genetically modified cells are injected into recipient embryos. As recipient embryos, freshly laid eggs are irradiated (6Gy - Cobalt source). Between 100 to 200 genetically modified cells are injected into the subgerminal cavity using a micropipet. The window in the egg shell is closed and the eggs are incubated. Somatic chimerism of hatched chickens is evaluated by PCR. Germ-line chimerism is assessed by 20 mating of somatic chimeras.

Example 15
Immunization Of Transgenic Animals

25 Genetically engineered chickens are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (5 μ g in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μ g/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk 30 (NFM) in PBS (300 μ l/well). Chicken serum is diluted in PBS/1%NFM and added to the

coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with HBsAg. At a dilution of 1:250 the optical density measured in uncoated and HBsAg coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with HBsAg. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-HBsAg antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (10 μ g in incomplete Freund's adjuvant) on day 0 and day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μ g/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μ l/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horse-radish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control serum from non-immunized rabbits is used. Serum from non-immunized rabbits does not react with HBsAg. At a dilution of 1:100 the optical density measured in uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized rabbits contains partially human antibodies reactive with HBsAg. At a serum dilution of 1:100 the measured optical density is 2.8. Upon further dilution of the serum the measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive

with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-HBsAg antibodies following immunization.

5

Example 16

**Complement Mediated Cytotoxicity of Virus Infected Cell Line
Using Humanized Antibodies**

A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi ⁵¹Cr in 100 ul PBS for 1 hr at 37°C. Two thousand ⁵¹Cr-labeled cells are incubated with serum from genetically engineered rabbits or chickens expressing anti-HbsAg humanized immunoglobulins. After two hours at 37°C the release of ⁵¹Cr into the supernatant is determined by measuring radioactivity using a scintillation counter. For the determination of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as follows: %Lysis = CPM experimental ± CPM#spontaneous / CPM# total ± CPM spontaneous. Incubation of labeled cells with serum (diluted 1:30) from non-immunized rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from immunized rabbits causes 80% cell lysis. Inactivation of complement in the serum by heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive. These results demonstrate that humanized antibodies produced by genetically engineered rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

25

Example 17

Immunization of Transgenic Animals against *Staphylococcus aureus*

Genetically engineered chickens are immunized intramuscularly with a recombinant fragment of the *Staphylococcus aureus* collagen adhesin protein (100µg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 µg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 µl/well).

Chicken serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with recombinant fragment of the *Staphylococcus aureus* collagen adhesin protein (100 μ g in incomplete Freund's adjuvant) on day 0 and day 14. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 μ g/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μ l/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized rabbit is used. Serum from non-immunized rabbits does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized rabbits contains

humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically 5 engineered rabbits produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Example 18

Protection Against *Staphylococcus Aureus* Infection In A Mouse Model

10

Naive mice are passively immunized i.p. on day -1 with 16 mg of the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein (from Example 17) or with the immunoglobulin fraction from non-immunized animals. On day 0, the mice are challenged i.v. with 4×10^7 CFU *S. aureus* per mouse and 15 mortality is monitored over the next 7 days. Mortality rate in the control groups is 80% and 10% in the group treated with the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein. The data indicate that anticollagen adhesin antibodies can protect mice against lethal *S. aureus* challenge.

20

Example 19

Antigen-Specific Hybridomas Made From Transgenic Animals.

Transgenic animals are immunized with an antigen (e.g., KLH, human red blood cells or sheep red blood cells). Spleen cells are removed at various times after 25 immunization and fused with myeloma cell lines derived from rabbit and chicken, respectively. After fusion cells are plated into 96 well plates and supernatants are tested for the presence of humanized antibodies. To demonstrate that the antibodies contain human immunoglobulin sequences, hybridomas are stained with fluorescent-labeled antibodies reactive with human heavy and light chain immunoglobulins. Limiting dilution 30 is conducted to purify hybridomas to monoclonality.

Example 20
Evaluation of Immunogenicity

5 Serum samples are collected from five cynomolgous monkeys on day 0. Subsequently, a purified partially human polyclonal antibody preparation (5 mg/kg) is administered into five cynomolgous monkeys by intravenous administration. The administration is repeated six times in bi-weekly intervals. Monkeys are monitored closely for any side-effects (e.g., anaphylactic shock, reflected by an elevated body
10 temperature). After seven months serum is collected from blood samples. Affinity resins containing purified human IgG or partially human IgG are produced by standard procedure using CNBr-activated Sepharose. Monkey serum samples (3 ml) are added to the IgG-affinity resin (4 ml) containing 10 mg human or partially human IgG. Subsequently, the columns are washed with PBS. Bound monkey immunoglobulin is
15 eluted from the column with 0.1M glycine/HCl pH2.5 and dialyzed 2 times against PBS. The protein content of the eluted fractions is determined using the BCA assay using human IgG as a standard. The total amounts of protein in these fractions demonstrate that therapy with partially human IgG does not lead to a significant antibody response in the treated animals.

20 **Example 21**
Treating Animals Using Humanized Antibodies

Humanized polyclonal immunoglobulins are purified from the serum of genetically engineered rabbits, or from egg yolk of genetically engineered chickens, by
25 ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are injected with one million human liver carcinoma cells expressing HBsAg. Subsequently, 25 µg immunoglobulin is injected peritoneally once per day. Animals treated with antibodies isolated from non-immunized rabbit serum die after about 60 days. This is similar to untreated recipients of liver carcinoma cells. In contrast, mice treated with
30 antibodies isolated from immunized rabbit serum survive for more than 150 days. This

demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

What is claimed is:

1. An isolated nucleic acid molecule comprising the sequence as set forth in any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, or a portion of any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13.
- 10 2. A recombination vector for replacing an Ig gene segment from a non-human animal with a human Ig gene segment, comprising from 5' to 3', a 5' nucleotide sequence, said human Ig gene segment, and a 3' nucleotide sequence, wherein said 5' nucleotide sequence and said 3' nucleotide sequence are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.
- 15 3. The recombination vector of claim 2, wherein said non-human animal is an animal which relies primarily on gene conversion in generating antibody diversity.
- 20 4. The recombination vector of claim 3, wherein said animal is rabbit, pig, chicken, sheep or cow.
- 25 5. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain constant region.
6. The recombination vector of claim 5, wherein said vector comprises from 5' to 3', a 5' nucleotide sequence as set forth in any one of SEQ ID NO: 12, SEQ ID NO: 13, a portion of SEQ ID NO: 12, or a portion of SEQ ID NO: 13; a human heavy chain constant region gene segment; a 3' nucleotide sequence as set forth in SEQ

ID NO: 10 or a portion of or SEQ ID NO: 10; and wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

7. The recombination vector of claim 5, comprising the nucleotide sequence as set forth in SEQ ID NO: 51 wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.
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8. The recombination vector of claim 5, wherein said vector is useful for replacing a rabbit light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 53.
10
9. The recombination vector of claim 5, wherein said vector is useful for replacing a chicken light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 57.
15
10. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain variable region.
11. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit heavy chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 52.
20
12. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit light chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 54.
25
13. A transgenic vector comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene
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segments is a human Ig gene segment, wherein said gene segments are juxtaposed in an unarranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

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14. The transgenic vector of claim 13, wherein said non-human animal is an animal which generates antibody diversity substantially by gene conversion.

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15. The transgenic vector of claim 14, wherein said non-human animal is rabbit, pig, chicken, sheep or cow.

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16. The transgenic vector of claim 13, wherein said humanized Ig locus is a heavy chain locus and comprises at least one V gene segment, at least one D gene segment, at least one J gene segment and at least one constant region gene segment.

17. The transgenic vector of claim 16, wherein said constant region gene segment is a human heavy chain constant region gene segment.

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18. The transgenic vector of claim 17, wherein said human heavy chain constant region gene segment is a Cy.

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19. The transgenic vector of claim 17, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

20. The transgenic vector of claim 19, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

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21. The transgenic vector of claim 13, wherein said humanized Ig locus is a light chain locus and comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment.
- 5 22. The transgenic vector of claim 21, wherein said constant region gene segment is a human light chain constant region gene segment.
23. The transgenic vector of claim 22, wherein said human light chain constant region gene segment is C λ or C κ .
- 10 24. The transgenic vector of claim 22, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.
- 15 25. The transgenic vector of claim 24, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.
26. The transgenic vector of claim 22, wherein said human V gene segment is placed immediately 5' to a J gene segment in a rearranged configuration.
- 20 27. A method of making a transgenic vector comprising a humanized Ig locus capable of producing a functional repertoire of humanized antibodies in a non-human animal, comprising:
 - 25 (i) obtaining a DNA fragment comprising an Ig locus or a portion thereof from said non-human animal which comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment; and
 - (ii) integrating at least one human Ig gene segment into said DNA fragment of step (i) to produce a humanized Ig locus, wherein said human Ig
- 30

gene segment is linked to the sequences of non-human origin operably as to permit gene rearrangement and gene conversion of said humanized Ig locus and the production of a functional repertoire of humanized antibodies in said non-human animal.

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28. The method of claim 27, wherein the integration of said human Ig gene segment is achieved by homologous recombination, thereby replacing an Ig gene segment in said Ig locus or said portion thereof from said non-human animal.

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29. The method of claim 28, wherein the homologous recombination is achieved in a bacterial cell, a yeast cell, or a non-human animal cell.

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30. The method of claim 28, wherein the human Ig gene segment is provided on a recombination vector, and is linked to a 5' nucleotide sequence and a 3' nucleotide sequence which are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

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31. A transgenic animal comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene segments is a human Ig gene segment, said gene segments being juxtaposed in an unarranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

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32. The transgenic animal of claim 31, wherein said animal is selected from rabbit, pig, chicken, sheep or cow.

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33. A B cell from the transgenic animal of claim 31.

34. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig heavy chains, comprising:

- (i) introducing a transgenic construct according to any one of claims 16-20 into a recipient cell of a non-human animal and integrating the humanized heavy chain locus in the transgenic construct into the genome of said recipient cell; and
- (ii) deriving an animal from the recipient cell having the humanized heavy chain locus integrated in the genome, thereby producing a functional repertoire of humanized Ig heavy chains.

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35. The method of claim 34, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

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36. The method of claim 35, wherein said rabbit has an impaired expression of endogenous Ig molecules.

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37. The method of claim 34, wherein said animal is chicken and said recipient cell is a fertilized egg.

38. The method of claim 37, wherein said chicken has an impaired expression of endogenous Ig molecules.

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39. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig light chains, comprising:

- (i) introducing a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal and integrating the humanized light chain locus in the transgenic construct into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized light locus integrated in the genome, thereby producing a functional repertoire of humanized Ig light chains.

5 40. The method of claim 39, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

41. The method of claim 40, wherein said rabbit has an impaired expression of endogenous Ig molecules.

10

42. The method of claim 39, wherein said animal is chicken and said recipient cell is a fertilized egg.

15

43. The method of claim 42, wherein said chicken has an impaired expression of endogenous Ig molecules.

44. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising:

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(i) introducing a transgenic construct according to any one of claims 16-20 and a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal, and integrating the humanized Ig loci in the transgenes into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized Ig loci integrated in the genome, thereby producing a functional repertoire of humanized antibodies.

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45. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising

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(i) making a transgenic non-human animal capable of producing a functional repertoire of humanized heavy chains;

- (ii) making a transgenic non-human animal capable of producing a functional repertoire of humanized light chains; and
- (iii) mating the transgenic non-human animal of (i) with the transgenic animal of (ii); and
- 5 (iv) selecting an offspring which produces both humanized heavy chains and humanized light chains thereby obtaining a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies.

10 46. A humanized immunoglobulin produced using the transgenic animal of claim 31.

47. A humanized immunoglobulin derived from a transgenic animal, comprising at least a portion of a human immunoglobulin polypeptide sequence.

15 48. The humanized immunoglobulin of claim 47, wherein said transgenic animal generates antibody diversity by gene conversion and/or hypermutation

49. The humanized immunoglobulin of claim 48, wherein said transgenic animal is a rabbit, chicken, sheep or cow.

20 50. The humanized immunoglobulin of claim 49, wherein said human immunoglobulin polypeptide sequence is a heavy chain or light chain polypeptide sequence.

51. The humanized immunoglobulin of claim 50, wherein said portion of a human immunoglobulin polypeptide sequence is a human constant region polypeptide sequence.

25 52. The humanized immunoglobulin of claim 51, wherein said human constant region polypeptide sequence is C γ , C κ , or C λ .

53. The humanized immunoglobulin of claim 51, wherein said portion of a human immunoglobulin polypeptide sequence further comprising a human V domain polypeptide sequence.
- 5 54. The humanized immunoglobulin of claim 47, wherein said humanized immunoglobulin is specific for an antigen.
- 10 55. The humanized immunoglobulin of claim 54, wherein said antigen is a microorganism selected from bacterium, fungus, or virus; an antigenic portion of said organism; an antigenic molecule derived from said microorganism; or a tumor-associated antigen.
- 15 56. The humanized immunoglobulin of claim 55, wherein said bacterium is selected from *S. aureus*, *Pseudomonas aeruginosa*, *enterococcus*, *enterobacter*, or *Klebsiella pneumoniae*.
- 20 57. The humanized immunoglobulin of claim 55, wherein said fungus is selected from *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, or *Cryptococcus neoformans*.
- 25 58. The humanized immunoglobulin of claim 55, wherein said virus is selected from respiratory syncytial virus (RSV), Hepatitis C virus (HCV), Hepatitis B virus (HBV), cytomegalovirus (CMV), EBV, or HSV.
59. The humanized immunoglobulin of claim 55, wherein said antigen is selected from Her-2-neu antigen, CD20, CD22, CD53, prostate specific membrane antigen (PMSA), or 17-1A molecule.
- 30 60. An antibody preparation, comprising the humanized immunoglobulin of any one of claims 46-55.

61. The antibody preparation of claim 60, wherein said preparation is a monoclonal antibody preparation.

5 62. The antibody preparation of claim 60, wherein said preparation is a polyclonal antibody preparation.

63. The antibody preparation of claim 62, wherein said preparation is substantially non-immunogenic to human.

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64. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and the antibody preparation of claim 60.

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65. A method of treating a disease in a human subject comprising administering to said subject a therapeutically effective amount of the antibody preparation of claim 60.

66. The method of claim 59, wherein said disease is caused by bacterial, fungal or viral infection, or said disease is a cancer.

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Figure 1(a)-(d). Novel nucleotide sequences 3'prime of the cow Cgamma gene (Cow Cy 3' flanking sequences). Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3, and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

clone11	1	50
clone3	CCTAAGACGTCGTGGTGATGCACGA	AACTTTACGG
clone5	CCTAAGACGTCGTGGTGATGCACGA	GGCCCTGCAC
clone11	151	200
clone3	AATCACTACA AAGAGAAGTC	CACCTCGAGG TCTCCGGGTA AATGAGCCTC
clone5	AATCACTACA CGCAGAAGTC	CACCTCTAAG TCTGCGGGTA AATGAGCCTC
clone11	201	250
clone3	GCGCCGCTGA TCTAGTGGAC	GTTCCCTCAT CCACCCACCC CTCCCCCAC
clone5	ACGTCCCTGC ACCAGCAAGC	CCTCACCCAG C..... ..CCACCCCTC
clone11	251	300
clone3	CCCAGGCTCC AGGTCCAGCC	AGGGCGCCCT AGCCCTCCCC TGTGTGCATT
clone5	CCCAGGCTCC AAGTCCAGCC	AGGACGCCCT AGCCCTCCCC TGTGTGCATT
clone11	301	350
clone3	CCTCCTGGGC CGCCGTGAAT	AAAGCACCCA GGCCGCCCTG GGACCCCTGCA
clone5	CCTCCTGGGC CGCCGTGAAT	AAAGCACCCA GGCCACCCCTG GGACCCCTGCA
clone11	351	400
clone3	ACGCTGTGCT GGTTCTTCC	GAGGCAGAGC CCTGGTGGCC GCCAGGCCCTG
clone5	ACGCTGTGCT GGTTCTTCC	GAGGCAGAGC CCTGGTGGCC GCCAGGCCCTG
clone11	401	450
clone3	CGGGGGTGGG CTGAGCCGAC	TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone5	CGGGGGTGGG CTGAGCCGAC	TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone11	451	500
clone3	GAGCTGACCC CACTCCGGGC	CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone5	GAGCTGACCC CGCTCCGGGC	CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone11	501	550
clone3	ACCTGGGGGC TGCCCAAGGC	CACAGAGGGG CTTGGCCAGA GGCACAGCTC
clone5	ACCTGGGGGC TGCCCAAGGC	CACAGAGGGG CTTGGCCAGA GGCACAGCTC

Figure 1(a)

clone11	CACGGTCCCC	TCCAGCCACC	ACCTGCTGGG	CCGGCCTCTG	GACAGGAACC	600
clone3	CACGGCCCCC	TCCAGCCACC	ACCTGCTGGG	CCGGCCTCTG	GACAGGAACC	
clone5	CACGGTCCCC	TCCAGCCACC	ACCTGCTGGG	CCGGCCTCTG	GACAGGAACC	
601						650
clone11	GGGAAAGCCC	CCGAGACCCCT	CAGGGATTGA	GGCCAATGC	TTCCCGCCTC	
clone3	GGGAAAGCCC	CCGAGACCCCT	CAGGGATTGA	GGCCAATGC	TTCCCGCCTC	
clone5	GGGAAAGCCC	CCGAGACCCCT	CAGGGATTGA	GGCCAATGC	TTCCCGCCTC	
651						700
clone11	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA	CATCCTTGTC	CCCAGGCC	
clone3	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA	CATCCTTGTC	CCCAGGCC	
clone5	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA	CATCCTTGTC	CCCAGGCC	
701						750
clone11	TGTCCTTGGG	TGTCCAGAGT	CCTTGTGTCC	ACTCTGGGCC	TGCCTGGAGC	
clone3	TGTCCTTGGG	TGCCCAGAGT	CCTTGTGTCC	ACTCTGGGCC	TGCCTGGAGC	
clone5	TGTCCTTGGG	TGTCCAGAGT	CCTTGTGTCC	ACTCTGGGCC	TGCCTGGAGC	
751						800
clone11	CACGCATGGC	CAGGGGGTGG	CCCTGCTTCA	CCCTCAGGCT	CCCAAGGTCA	
clone3	CACGCGTGGC	CGGGGGATGG	CCCTGCTCCA	CCCTCAGGCT	CCCAAGGTCA	
clone5	CACGCATGGC	CAGGGGGTGG	CCCTGCTTCA	CCCTCAGGCT	CCCAAGGTCA	
801						850
clone11	GGCCTCGCCC	TCCCTCGGCC	AGGAGGCTCT	GCCCGGCTCT	CCCTGCCAG	
clone3	GGCCTCGCCC	TCCCTCAGCC	AGGAGGCTCT	GCCCGGCTCT	CCCTGCCAG	
clone5	GGCCTCGCCC	TCCCTCGGCC	AGGAGGCTCT	GCCCGGCTCT	CCCTGCCAG	
851						900
clone11	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA	TCCCTGTGCC	TGAAAGGGT	
clone3	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA	TCCCTGTGCC	TGAAAGGGCT	
clone5	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA	TCCCTGTGCC	TGAAAGGGT	
901						950
clone11	CCAGGCGAG	AGCCCTGAAT	GTCCAGGGCA	GGGACCTAGC	TGCTCCCTGT	
clone3	CCAGGCGGG	AGCCCTGAAT	GTCCAGGGCA	GGGACCTAGC	TGCTCCCTGC	
clone5	CCAGGCGAG	AGCCCTGAAT	GTCCAGGGCA	GGGACCTAGC	TGCTCCCTGT	
951						1000
clone11	GGACACGGAG	CCCAGAGCCA	CAGACAACAA	GCCCCAGCCC	CGCACGCACA	
clone3	AGACACGGAG	CCCAGAGCCA	CAGACAACAA	GCCCCAGCCC	CGCACGCACA	
clone5	GGACACGGAG	CCCAGAGCCA	CAGACAACAA	GCCCCAGCCC	CGCACGCACA	
1001						1050
clone11	CGAGACAGCC	CGCACCCAGC	CTCCTCCACA	CGCACTCAGG	TGTACATGCG	
clone3	CAAGACAGCC	CGCACCCAGC	CTCCTCCACA	CGCACTCAGG	TGTGCATCCG	
clone5	CGAGACAGCC	CACACCCAGC	CTCCTCCACA	CGCACTCAGG	TGTGCATCCG	

Figure 1(b)

clone11	CACATGAGCA CACTTCACCC CGTCACACCC ACACACCTAC ACACACTCAG	1051	1100
clone3	CACATGAGCA CACTTCACCC CGTCACACCC ACACGCCTAC ACACACTCAG		
clone5	CACATGAGCA CACTTCACCC CATCACACCC ACACGCCTAC ACACACTCAG		
clone11	GTCTCGCACT CGGGGGACCCA TGGGGTGACC CCACGGGCC AGA.CCAGAG	1101	1150
clone3	GTCTCGCACT CGGGGGACCCA TGGGGTGACC CCACAGGCC AGACCCAGAG		
clone5	GTCTCGCACT CGGGGGACCCA TGGGGTGACC CCACAGGCC AGACCCAGAG		
clone11	CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGGCC CCACCCCTCCA	1151	1200
clone3	CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCCTCCA		
clone5	CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCCTCCA		
clone11	GCGCCCATGG GCTGCTCAGC GGCCCTTTCC CACACTGACC ACACTGACCA	1201	1250
clone3	GCGCCCATGG GCTGCTCAGT GGCCCTTTCC CACACTGACC ACACTGACCA		
clone5	GCGCCCAGGG GCTGCTCAGC GGCCCTTTCC CACACTGACC ACACTGACCA		
clone11	GGTCAGACAT CGTTCCTTG CCTCCCCCTGG GACACCCACG CCCCTCCCTA	1251	1300
clone3	GGTCAGACAT CGTTCCTTG CCTCCCCCTGG GGCACCCACG CCCCTCCCTA		
clone5	GGTCAGACAT CGTTCCTTG CCTCCCCCTGG GGCACCCATG CCCCTCCCTA		
clone11	GCAGGCTGAG ATCCCCCCCCTC AGCCCCCTCGT CCTGGCAGCC TCACCCCTCG	1301	1350
clone3	GCAGGCTGAG ATCCCCCCCCTC AGCCCCCTCGT CCTGGCACCC TCACCCCTCA		
clone5	GCAGGCTGAG ATCCCCCCCCTC AGCCCCCTCGT CCTGGCACCC TCACCCCTCA		
clone11	GGCACAGCAC CCCTCAGGCC CGGTGCTGTC AGCCCTCCCT CCCGGGGGC	1351	1400
clone3	GGCACAGGGGA CAC...AGCC CGGGCTGTC TGCCCTCCCT CCCTGGGGGC		
clone5	GGCACAGGGGA CAC...AGCC CGGTGCTGTC TGCCCTCCCT CCCTGGGGGC		
clone11	AGGGCCCAGG AACGTGCGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC	1401	1450
clone3	AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC		
clone5	AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC		
clone11	CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCTGT GGGGAGGTAG	1451	1500
clone3	CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCTGG GGGGAGGTGG		
clone5	CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCTGG GGGGAGGTGG		
clone11	GGTCAGGGTC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACCCAC	1501	1550
clone3	GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAAC AGGACACCCAC		
clone5	GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACACCCAC		

Figure 1(c)

	1551	1600
clone11	AGGCCAGGCC	CAGATAGCAG CCAGGGCTGG AGGGGTTGGG GCTGGGGCTG
clone3	AGGCCAGGCC	CAGACAGTGG CCAGGGCTGG AGGGGTTGGG TCTGGGGCTG
clone5	AGGCCAGGCC	CAGACAGTGG CCAGGGCTGG AGGGGTTGGG TCTGGGGCTG
	1601	1650
clone11	GGCCCCAGAG	ACTGACCTCA GGTGACCCCT GCCTGGCCCA TGGGGAGATC
clone3	GGCCCCAGAG	ACTGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC
clone5	GGCCCCAGAG	AATGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC
	1651	1700
clone11	ACGCCACCTT	CCCCCCACCC AGAGGGAGCC CTGCCC...T ACCCCAGTGA
clone3	CTGCCACCTT	CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA
clone5	CTGCCACCTT	CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA
	1701	1750
clone11	CCCTGCCAG	CCCTCCGTGG GCAGACACAG CACTGACCAAC CCCTCCCTGT
clone3	TGCCACCCAG	CCCCCCGTGG GCAGACACAG CACTGACCAAC CCCTCCCTGT
clone5	TGCCACCCAG	CCCCCCGTGG GCAGACACAG CACTGACCAAC CCCTCCCTGT
	1751	1800
clone11	GCAGACTTGC	TGCTGGAGGA GGAGATCTGT GCGGACGACC TGGATGGGGA
clone3	GCAGACCTGC	TGCTGGAGGA GGAGATCTGT GCGGACGCC AGGACGGGGA
clone5	GCAGACCTGC	TGCTGGAGGA GGAGATCTGT GCGGACGCC AGGACGGGGA
	1801	1850
clone11	GCTGGACGGG	CTCGACCGACGATCGAT GCTG
clone3	GCTGGACGGG	CTCGACCGACGATCGAT GCTG
clone5	GCTGGACGGG	CTCGACCGACGATCGAT GCTG

Figure 1 (d)

Figure 2(a)-(e). Novel nucleotide sequences 3'prime of the sheep Cgamma genes. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

clone11	101	150
	GCTAAGCCCTG TCTGGTGTGTC	
clone11	CACGAGGCTC TACACAACCA CTACACACAG	
clone1	CCTAGGCCCTG TCTGGTGTGTC	
	151	200
clone11	AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACATGCC CCCGCACCAG	
clone1	AAGTCGGTCT CTAAGCCTCC GGGTAAATGA GCCACACGCC CCCGCACCAG	
	201	250
clone11	CAAGCCCTCA CCCAGCCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG	
clone1	CAAGCCCTCA CCCAGCCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG	
	251	300
clone11	CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA	
clone1	CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA	
	301	350
clone11	CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTCT TTCCGAGGCA	
clone1	CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTCT TTCCGAGGCA	
	351	400
clone11	GAGCCCTGGT GACCGCCAGG CCTGCGGGGG GTGGGCTGAG CCCACTCTGG	
clone1	GAGCCCTGGT GATGCCAGG CCTGCGGGGG GCAGGCTGAG CCCACTCTGG	
	401	450
clone11	GCCGCTTGGT TCAGCATCTG TGGGGCGCT GACCCCTCTC CGGGCCAGAC	
clone1	GCCGCTTGGT TCAGCATCTG TGGGGCGCT GACCCCTCTC CGGGCCAGAC	
	451	500
clone11	ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCC GAGGCCTCGG	
clone1	ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCC GAGGCCTCGG	
	501	550
clone11	AGGGGCTTGG CCAGAGGCCGC AGCTTACGG CCCCTCCAG CCACCACATT	
clone1	AGGGGCTTGG CCAGAGGCCGC AGCTTACGG CCCCTCCAG CCACCACATT	
	551	600
clone11	CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA	
clone1	CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA	
	601	650
clone11	TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG	
clone1	TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG	

Figure 2(a)

clone11	651	700
	GCCGCGGCCT	TGTCCCCAGG CCCCTGTTCC TGGGTGCCA GAGTCCGTGT
clone1	GCCGCGGCCT	TGTCCCCAGG CCCCTGTTCC TGGGTGCCA GAGTCCGTGT
clone11	701	750
	GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC	
clone1	GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC	
clone11	751	800
	TTCACCCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT	
clone1	TTCACCCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT	
clone11	801	850
	CTGCCTGGCT CTCTCTGCCG GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT	
clone1	CTGCCTGGCT CTCTCTGCCG GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT	
clone11	851	900
	CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG	
clone1	CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG	
clone11	901	950
	CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC	
clone1	CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC	
clone11	951	1000
	AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA	
clone1	AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA	
clone11	1001	1050
	CACGCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA	
clone1	CACCCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA	
clone11	1051	1100
	CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG	
clone1	CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG	
clone11	1101	1150
	TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG	
clone1	TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG	
clone11	1151	1200
	GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC	
clone1	GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC	
clone11	1201	1250
	TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC	
clone1	TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC	

Figure 2 (b)

	1251		1300		
clone11	CTGGGGCACC	CACGCCCTC	CCTCGCAGGC	TGAGACCCCC	CCTCAGCCCC
clone1	CTGGGCACC	CACGCCCTC	CCTTGAGGC	TGAGACCCCC	CCTCAGCCCC
	1301		1350		
clone11	TCGTCCTGGC	ACCCTCACCC	CTCGGGCACA	GGGACACAGC	CCGGCACTGT
clone1	TCGTCCTGGC	ACCCTCACCC	CTCGGGCACA	GGGACACAGC	CCGGCACTGT
	1351		1400		
clone11	CTGCCCTCCC	TCTCGGGGAC	AGAGCCCAGG	CACGTGTGCT	CTGCTGAGCC
clone1	CTGCCCTCCC	TCTCGGGGAC	AGAGCCCAGG	CACGTGTGCT	CTGCTGAGCC
	1401		1450		
clone11	TCCCCGGCTCC	AGGCCTGGCC	CCCAGGGCAG	AGGAGGCCAG	GAATTGAGCC
clone1	TCCCCGGCTCC	AGGCCTGGCC	CCCAGGGCAG	AGGAGGCCAG	GAATTGAGCC
	1451		1500		
clone11	TCTGTCCTGC	GGGGAGGTGG	GGTCAGGGCC	CCAGCTCAGG	GCACAGCTCA
clone1	TCTGTCCTGC	GGGGAGGTGG	GGTCAGGGCC	CCAGCTCAGG	GCACAGCTCA
	1501		1550		
clone11	GGATGGGAGC	AGGACCCCAC	AGGCCAGGCC	CAGACAGTGG	CCAGGGCTGG
clone1	GGATGGGAGC	AGGACCCCAC	AGGCCAGGCC	CAGACAGTGG	CCAGGGCTGG
	1551		1600		
clone11	GGCTGGGGCT	GGGGCCCAGA	GACTGACCTC	AGGTGACCCC	TGCCCCGGCCC
clone1	GGCTGGGGCT	GGGGCCCAGA	GACTGACCTC	AGGTGACCCC	TGCCCCGGCCC
	1601		1650		
clone11	ATGGGGGATC	ACACCGCCAT	CCCCCGGCC	GCAGAGGGAG	CCCTGCCCG
clone1	ATGGGGGATC	ACACCGCCAT	CCCCCGGCC	GCAGAGGGAG	CCCTGCCCG
	1651		1700		
clone11	AAGCCCCGAT	GGCCCCGCC	AGCCCCCGT	GGGCAGACAC	AGCACTGACC
clone1	AAGCCCCGAT	GGCCCCGCC	AGCCCCCGT	GGGCAGACAC	AGCACTGACC
	1701		1750		
clone11	CCCCCTCCCTG	TGCAGATCTG	CTGCTGGAGG	AGGAGAGCTG	TGCGGACGCC
clone1	CCCCCTCCCTG	TGCAGATCTG	CTGCTGGAGG	AGGAGAGCTG	TGCGGACGCC
	1751		1800		
clone11	CAGGACGGGG	AGCTGGACGG	GCTCTGGACG	ACTATCTCCA	TCTTCATCAC
clone1	CAGGACGGGG	AGCTGGACGG	GCTCTGGACG	ACTATCTCCA	TCTTCATCAC
	1801		1850		
clone11	GCCCTTCCTG	CTCAGCGTCT	GCTACAGCGC	CACCGTGACC	CTCTTCAAGG
clone1	GCTCTTCCTG	CTCAGCGTCT	GCTACAGTGC	CACCGTGACC	CTCTTCAAGG

Figure 2 (c)

	1851					
clone11	TGGGGGTCCA	CCCTGCTGGG	CCCTCGGGCC	CCCTCTCTGT	CCCCAGGGTC	1900
clone1	TGGGGGCCA	CCCTGCTGGG	CCCTCGGGCC	CCCTCTCTGT	CCCCAGGGTC	
	1901					1950
clone11	CCCGCAGAGT	CCCTCCCTGC	CCCTCACTGT	CCCTCCCTGT	CCCTCTCTGT	
clone1	CCCGCAGAGT	CCCTCCCTGC	CCCTCACTGT	CCCTCCCTGT	CCCTCTCTGT	
	1951					2000
clone11	CCCTCTCTGT	CCCTCTCTGT	CCCTCTCTGT	CCGTTCATTT	TCCCTTCACC	
clone1	CCCTCTCTGT	CCCTCTCTGT	CCCTCTCTGT	CCGCTCATTT	TCCCTTCACC	
	2001					2050
clone11	GTAAGCTTGA	GACAGATTGG	GGTCATTTCA	GAGGGCGTCT	GAAGAGTCTC	
clone1	GTAAGCTTGA	GACAGATTGG	GGTCATTTCA	GAGGGCGTCT	GAAGAGTCTC	
	2051					2100
clone11	TGTGCCGCAC	GCCTCCCTTC	ATGTCAGTGG	GGAGAATTCA	GCAAGGGTGG	
clone1	TGTGCCGCAC	GCCTCCCTTC	ATGTCAGTGG	GGAGAATTCA	GCAAGGGTGG	
	2101					2150
clone11	AGTGCTGGGT	GAGAAATGAG	GCTTGCGGCG	CTCACGAGCA	GTGATGGGGC	
clone1	AGTGCTGGGT	GAGAAATGAG	GCTTGCGGCG	CTCACGAGCA	GTGATGGGGC	
	2151					2200
clone11	ACTGCTGCTC	CCTGAGACCT	GCGCGGACAC	CGTTTTCCAT	CGCAGGAGAA	
clone1	ACTGCTGCTC	CCTGAGACCT	GCGCGGACAC	CGTTTTCCAT	CGCAGGAGAA	
	2201					2250
clone11	GCGGGCAAGG	GAAAACGCC	TCTTGGTCTC	TCTTGAGTAA	ATGTCGCGTT	
clone1	GCGGGCAAGG	GAAAACGCC	TCTTGGTCTC	TCTTGAGTAA	ATGTCGCGTT	
	2251					2300
clone11	TTGGTCATCA	GTCCCTCCCC	CAGTGAGGCT	AGAGGAGTTT	ACTTCTCCCT	
clone1	TTGGTCATCA	GTCCCTCCCC	CAGTGAGGCT	AGAGGAGTTT	ACTTCTCCCT	
	2301					2350
clone11	CTCGATGGTC	AGGTCAAGGAC	TGTCATAGAC	TCCGGATCAC	CTTCCTGTAA	
clone1	CTCGATGGTC	AGGTCAAGGAC	TGTCATAGAC	TCCGGATCAC	CTTCCTGTAA	
	2351					2400
clone11	ATGCTTGCTT	TTTGTGTGCA	GAGAGCCTGT	TTTAGCTCGG	GGGTCCCTCAG	
clone1	ATGCTTGCTT	TTTGTGTGCA	AGAGCCTGT	TTTAGCTCGG	GGGTCCCTCAG	
	2401					2450
clone11	CTCACTGAGC	TCGCGGGGCA	GGGGTGGGCT	CGGGCTGGCG	CCGCCTGTTC	
clone1	CTCACTGAGC	TCGCGGGGCA	GGGGTGGGCT	CGGGCTGGCG	CCGCCTGTTC	

Figure 2 (d)

Figure 2 (e)

Figure 3(a)-(b). Novel 3'prime flanking sequence (SEQ ID NO: 10) of rabbit Cgamma gene.

TGTGCACCCCCCACACAAATAAAGCACCCAGCTCTGCCCTGAGAGGCTGTCTGATTCC
 TCCAAGGCAGAGGCTTCACTCGGCCGGACAGGGTTGGCGGGCGGTGGCTCTGCT
 GTGGCCAGCAGCCAGAACGGTCAACAGTGGACAGGGCAGACCCACAGCACAGGGCCT
 GCCAAGAACTGGGCTCAGCCGGAGTGTCTGGCAGGTCCCCCTTGCAGCTAGCACGTGT
 GTGCTGGCAGGCAGAGGGCCCGAGGGGAGGAGCACAGCTACCACTCTGCAAGAGCC
 TGGCCTGGCCAGGTCCAGTCCACAGGGTGTGTAGTACACAGAGCCTCATCTTACCA
 CAGATGTAGGGACAGACCCACCACGCCCTGCACCCACCCAGGCTCGCCCCCTGTGGGA
 CCAGGGCTTACCACTCCACTCCCCGCCAGAGCAGCAGAAGCAGGTGGCATCCTCAGCAG
 AGGGACAGTCTACCCCTCACGGCAGTGAGCCCTGACCCATCAAACAAGCCCTCCTGC
 TGACACAGCACCTGTGTGCACATCACACACACACACACACTGAGGCTGACCCCA
 TCAAACAAGCCCTCCTGTCACAGCACCTGTGTGCACATCACACACACACACAC
 ACACATGAGGCCCTGACCCCATCCTGCCCTCCTGCTGCATGGCACCTGTGTGCACATCACAC
 ACACATGCACACACACACTCACACACACTGAGCCCTGACCCATCCTGCCCTCCTGCTGC
 ATGGCACCTGTGTGCACATCACACACACACACACACACACTGAGCCCTGACC
 CCATCCTGCCCTCCTGTCATGGCACCTGTGTGCACACATCACAAACACGCCCTGCTCATA
 CACTGGCACTCAGAAGGGGCCCTGTACACGCATCACATGCACACACCTTGACACATGG
 GCCCCCTACACACGCATCACACACACTCATGCACACTCCTCACACATGCCCTCCTGAC
 ATACATTGCACACACATGTGCACAGCCTCACAAATGGCCCTGCAACACACATTGTACAC
 ACGCATGTGCACACACTTCACACATGGCCCCCTGCACATGCATTGCACACACAGACACA
 CACATGTGCATTCCCTCACACATTGGGCCCTTGCAAGGGATGCCCTGCACACACATTGCAC
 ATGCTCACATGTGCACACACCCACACTGGAGCCTGCAAGGGCCCTGACATGCACATCAAACACATATG
 CATGCATAACACACACACCTCACACAAGGGCCCTCATACAGCAGGACACACACAC
 CATGCACACACCTCATACAGGGGCCCTACACACATCACACACACACACACAC
 ATGCAAGGGCCCTTGTACACACACTGCAAGGGGCCCTGACATGGCACACATGTGTACATGCT
 TCACACACTGGGCCCTTGCAAGGGGCCCTGCAAGGGGCCCTGCAAGGGGCCCTGCAAGGGGCCAG
 GTGCTGGCACATGGACACTGGTGCACACACAGCACCCAGCTCTCCATCCAA
 GGGGACCCAGCACCCCTACACAGCACCTGAATTCTGCTCCCAACAGCAACGT
 GCACCTACCTCTCCAGACGCTCCCTTGCAAGGGGCCCTGACATCCCATAAGGTATTGGCAGACC
 CTCCCTGACCCCTGGGCCCTGGTCACCCAGGGACAGGGAGAGGGCAAGTTGGGCCACAG
 TACCACTGCCAGCAGGGTGAAGGCAAGCAGAGGGTGGTCTGTGAGGCGTCTGGCCAGC
 CGTGTGGGCCAGGTGGGGAGCAGTGGGTGGCTGAGGTGGCTTCTTGCAAGGTGGTT
 GGGGGAGCTGGCCCACAAAGTGCACACTGCCAGCTGTCCAGTGCTTCCCTGAAACC
 TCCCGGCCACCCATCCAGCTGCAGCCAGAGGGAGTGGCCCTCCTGGCCTCTGGCAA
 GACGCACGCTGACTGCCCTCCCATCCAGAGCTGCAGCTGGACAGAGAGCTGTGCGAGG
 CCCAGGACGGGAGCTGGACGGCTGGACCCACATCCACCATCTTCATCTCCCTCTTCC
 TGCTCAGCGTGTGCTACAGGCCACAGTCACCCCTCTCAAGGTGGTGCTGCACCCGGCA
 CGGGTGGCTGGGGCCAGGGCGGGGGCCAGGGCCCTCCTCACCCCGGCCGGC
 CGCTGCTGCAGGTGAAGTGGATCTCTCGTCCGTGGTGGAGCTGAAACACACCATCGCTC
 CCGACTACAGGAACATGATCGGGCAGGGGCCCTAGGCCCTTCGTTCTCACAGCCTGCCTC
 CCTGCCAGCAGGAGGCCCTCCGCTCGGCCAGGCCATGGCTCTGCTCTGGCCGCT
 CGGACCCCTCCGCTCGGGAGAAGCGCCAGCTGATGCCCTGCCCTCCACGCAGC
 AGTGCAGCAGCACGCTGTCGTCACCCGGCAGGACCCACCCAGGGCAGCCCTGA
 CGCCAGCCTCTGGACTCAGGGCTCCTTGAGAAAAGGCCACTTGTGGTCCCTCAG
 CCCACACCCAGGCAGCCTCCGGTGGGTGCTCCCTGACCCAGGCTGAGGCTATGCTT
 GTTCTCTGTGGCTTACTCAGAGGCCGTGCTGGACTCCACCCACAGGGACAGTGCC

Figure 3(a)

CTGCTCCAACCCCTCACTGCACTGGGGTCAATGGGGCACCTCTGTGCAGGGGTCTGGC
TCCAAGGAGAACACTCGAAGGGCCTGCTTGGCCACCTGGCACACGGGAGCCCCGCTGGG
TAGCTTGGCAGGGACCCCTGAGTAGAGGTGGGTGCACCCAGCCAGAAAGCCTGCTGGATG
GACAGGGAGCCTGGCGTCCGGCCCCAGGCAGGCAGACACGGCTTCATGGACAGGAGAGGC
CAAGGAACATCAGCAAAGAGAGACAGCTGGCCGGCGTTCCAGCCAGACCCATCCTGCA
GCCAGCATGGCCTCACTGACCCAGCCACAGGAG

Figure 3 (b)

Figure 4. Novel nucleotide sequence (SEQ ID NO:11) 3'prime of the rabbit Ckappa 1 gene.

GGCCAGCCTCGCCGCTCCCTCCCTCAGTGGACCCATTCCACACAGTCCTCAGCCCC
TCCCCCTCCCGGCCCTACCCCCCTCTGGCTTAACCTTGCAGATGTTGGTAGAGATGGAT
GAATAAAGTGAATTTGCACTTGTGACTTCTCTGCTTCTTCATTATGGTTATTAC
TCATGTTTCCCAGTTGCCCTAAAGTCACCGCATTCTCATCCTCCATCCACCCCTGCCCT
GCTGTCCTCCGGGAGACACCACCTCCCTGAAACCCACAGGCCCCGTCTTCACACCGCCGA
CCCCGACCACACGTGAGGGGCTTGCTCGTCTACTCCCCCATCGAGCCCCAGAGTC
CTCCTTGTCTTACAGTACATACAGTTACAGTTGAGTCATCCAACTGCC
TGCCAAATTCCCCAAAACAAGATTTCAGAATAAAACAGCTATGAGAAAAGTCATTATG
GAAGCATGATATAACAAACAAAACAATGCAAACAACTTAACGATAAGCAGAGGGAAA
TGTTCAGACACACTATGGGGCTTGGGCTTCATGGAGTATTACACCTTCATTACATT
AACTTGTTATTAGGAGCTCTTACAGGATTACTAGAGCACTTCCATGACCTA
ATTAATTCTCATACACTGTGAGGTTAAAGCATTAGTTAAATATTGGCAGGCTCCCT
ATAGCCAACAGTTGTTCATATTCCATAACCCAACCATCATTAGGTGACTCAGGGCTCCTT
GTCCACCAAGAACCTTGGCAAGAATGTTGAGACAACTCCCTTATAAAAGTCAAAATT
GGAAGTAACCTAAAGTCTACCAACAGTAGAATGGGCTTAAATTGGCATATGTTACAT
ATTAGAATGCTGTTAATAAAAGAGAATTAAACAAACTACACTATCCCTAATAACATAGGT
GACTCATAAACATGATGTTAAGCACAAGAACCCAAACACAAAAGACACACTGTTGATGTT
TTCATCCATAGGAAGTTCAAAACTAGTTAAAATTGAATTAGAAATTGAGATGAAAGTT
CTCTGGCTGGGGGTGTGGAGTGAGCGGTGCTGGTGGGGACAGAAAGTGGCTGCTGG
GGTCTGGTGTGTTCTAGTCCACTGTGGTGTGCTACTCTGAAATGTATTGAGTA
ACAATAGGTTTGTGTTTCAATTACTCCAAAGTAAGTCTCATAAACATTGCCCTA
CACGGGGTCTACAGATAAGAGAGACTAAGAGGAATGAGTAACAGATCAAGGCCACACAGC
TGGTAGGCATGGGCTGGGATCAAACCTGTCTGCCAATTCTGCTCTTGAGGCCCTA
ACTATCTTCCAGCACTGGAATGCCATGAGAACAGGGAGTAGGACATGCTACCTCCCT
AGGGTCTCCTCTTACCCACCTAACCAACAGGAGCACCCATACATAGAAACAGGATGGAAA
GACCATCAGCAATGAAACAAGGGAGAGATTAACCTGTCAGTATTGTGATCCCCATGTC
GAAAGATTGTTGGAGGGCTGCACACAGAGCACCGTCCCCCTCTATGTGCCACCGC
TCTGTGCCCCCTATCTGTCACCCGCCAGCGTGCATTCACTCAGCACCCCTTCGCCT
GCCCTCTGAAAGAGGTTGAGAAGTAACTAACCAGCTTCCCTCTTCAGTGAATTGGAAAT
CCAGTTTCCCTCTTACCTTGGGCTTCACTCTGTCAGTGCAGGAGCCTGGAGAAATGTGATT
TGTGTTATTATAAAATTCTCCACATCATTGTGTAAGGAAAATATACTCAACAGTCATA
ACTGGTAAACTGCTGTGAAACTAAGAGAGTAATTGCAAGGGTTGAGCACCAGCC
TTGTATATACTAAGAGATCCAGAAGTGTGTTGCAACCGTTAGAAATAAGAAGGAGTAGCTC
AATTGACTAGTTCCTGGTTCACCTCTGTCAGTGCAGGAGCCTGGAGAAATGGTAAAT
CAAATGATTGAACTTGGAAATTAACTCACATGGATTCTAGACCTGTGCCGAGAATGGCTGC
CACTCGTGTCTAGAGCTCTGGGATGAGGTGCTCCCTACTGTGGTGTGCTACAGGTCTA
ACAACACACCAAGGTTTGAAGACTTAGCACTATGAAATAATATATATATTATTCACAT
AAATTAAACATACTTCTACTTCAATTGCACTGTTGAGATAGTAATCTACTTGGATATAT
TTGGTTAAACCAAACATTCTCAAGACAAATTCTCATGGTTTATGGTTTTTCAAAATT
AAATCAAATATAAAACATAGTCCAAACAACTTAATCCATTAAAGTGGAGAATGGCCAAGT
GTTTGGGCCCTGCTACCCATTGAGACAGATGTTGCTCTGGCTCTGGCTTTG
TTGGCTAGCCCTGGCCATTGAGCCATCTGAGGAGTAACAGTGGATGGAAGACATCTC
GGCCACCCGCTGGGCTTGTGAGGAGTAGCTGAGGAGTAACAGTGGATGGAAGACATCTC

Figure 5. Novel nucleotide sequences (SEQ ID NO:12 and SEQ ID NO: 13) 5'prime of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

CTTGACGCCGAACTGCGCCCTCCCGCTGAGTCTCACCGCACCCCCCG
GTGCAGGCCAGTCGGCCTCACCTGGAAGGTGACTGACTGAAGACACTGCAAGGGGTGAG
AGCATTTCAGGAAAGAGCCCTGAGTTAGAAGGCCAGAGAGCAGAGGGCTGAGGGCTG
CCTTGCCTGCAACCCATGAAACACAGGCTTAGCAGATGTTCAAGCTCCGGAGTCCAC
ACTGGGTGAGGGCAGGCGTCAGCCTGACATGGCCCCACAGACTCGCCCACAGGTGACG
CCAGATGAGGACGGTCAAGGATGGGGATCCTACATGCCAGGGCACCAAGACAGCCA
GGAGAGCACCAAGAGGCCAAAGAGAGGCCAGTCTCCCTGCTGACATCCAGAGCC
CAGGCCCACTTGCAGACTGGCTGAGAACACGTCCTGCGGTGGAAGCTGCCCGTCC
TGGGTGTTGCTGGGGCTAAGCCGACTGACGCCAGGCCCCAGGCCATGGCCCCAC
GGCCTGCAGCTTCTCCCCAGGCCACGTGGCTCTGGCTGAACCTGGCGCTCGC
TGAGCTCTACCCCCCCCACCCAGCAGCAGGCCGGGCGGTGCTGCCATGAGCTCCATTCCC
ACCACACAAGCGACAGCCCGGGCAGCAGCCCCACGGCCACGGGGCTTGTGCGGCTC
GCACTCGCTGCTCAGGGCCAGCGCAGGGTGCAGCAGGGACTCACCAACCCGCCCCGACTC
GGCTGGCACGTTACTGGAGGCCTGAGCCTGACCGTGGCAGTGGGGCCGAGCAGGCT
CCAGGCTGCCCTGACCCCTGGCTTGGCCTCCGGGCTCTGGGCTCATAGCGGTCACTCTCCGCTTC
GATGTCCTCCCACCGCTTCTGGCTCTGGGCTCATAGCGGTCACTCTCCGCTTC
TCTCCTCCCAGCCCTTCTGGCTCTGGGCTCATAGCGGTCACTCTCCGCTTC
CTCTACCTGGAAGGAATCTGCTGTTGGACCAAGACACCCCGCAGCACAGGTGGCGCC
TTGCACTGTCCTGGGCTCCCGCACAGAAAAGGGCCCTAGGCTCTGGAGGGCTGCTGCT
GNCTCTGGGCTGGCATCGGGCGACCCCTGCACCCCTGAGGAAACTCAGGCCTG
CCCGCTCCAGGCCCTGTCCT

Gap of about 1000 nt

GAAGCTTACTTGTGGGGCCGG
GCAGGTCTAAGGGACCTGCCAGGTGTGGGGCTGGGCTTGACTCAGCAGGAGCCTCTAG
AAGGAAAGCTCTGGAGAAGGTGGGGCAGAGGGGGAAAGGCCCTGTGAGGAGGGGGTAG
GTGGGCAAGGCCACTGGGAAGGGAGGGCTGGGGTGAACACTCAGGTTGGCACTGGGAGG
ACCTGAGGAGGCAGGTGCCAGGCACAGACTGAACCTGGGCAAGGGCAGGGCAGGTAACA
AGAAGGATTCTCTGGAGCCTGGTCCAGGGTGTGTCAGGGCGGTCCAGGGCTGGGTT
TGCAAGCTGGGCTGTGACAGGGCTCTCTCCAGGGCAAGCAGCAAAGCCTGGGACA
GAGCCAAAGCCCCACACAGAGAAGCTCCCAAGGGCAGGGCTGCAGGGCTGGGGAC
CTTCTGGAGCAGGCAGGGACAGAGGCATGAGATCAGCCTCCAGAGGCTGGAATGATA
GGTCCAGCAGGAGGGCCCACATGGCTCTGGTAGCAGGAGAAAACAGCCCCCAGGTCC
CCATGCCACCAACCGACTGCTGGTGAACCTTGGGTGGCAGACGAGAGGCCACATGG
CAGCTGCTCCTGTCAC

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the the M1 and M2 regions 3' of the Cgamma gene.

M1

	1	46 SEQ
camel	EPLLEESCA EAOSGELDGL WTTISIFITL FLLSVCYSAT VTLFK.	14
human-Ig3	.ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK.	15
human-Ig3/2	.ELQLEESCA EAQDGELDGL WTTITILITL FLLSVCYSAT VTFFK.	16
human-Ig1	.ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK.	17
mouse-Ig1	.GLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK.	18
mouse-Ig2a	.GLDLDDVCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK.	19
mouse-mRNA	PGLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK.	20
mouse-Ig3	.ELELNETCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK.	21
mouse-Ig3/2	.ELELNGTCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK.	22
sheep-clone11	.LLLEEEESCA DAQDGELDGL WTTISIFITP FLLSVCYSAT VTLFK.	23
sheep-clone1	.LLLEEEESCA DAQDGELDGL WTTISIFITL FLLSVCYSAT VTLFK.	24
cow-clone11	.LLLEEEICA DDLDGELDGL	25
cow-clone3/5	.LLLEEEICA DAQDGELDGL	26
rabbit	..LQLDESCA EAQDGELDGL WTTITIFISL FLLSVCYSAT VTLFK.	27

M2

	1	27	SEQ
camel	VKWIFSSVVE LKRTIVPDYR NMIGQGS		28
human-Ig3	VKWIFSSVVD LKQTIIIPDYR NMIGQGA		29
human-Ig3/2	VKWIFSSVVD LKQTIIIPDYR NMIGQGA		30
human-Ig1	VKWIFSSVVD LKQTIIIPDYR NMIGQGA		31
mouse-Ig1	VKWIFSSVVE LKQTLVPEYK NMIGQAP		32
mouse-Ig2a	VKWIFSSVVE LKQTISPDYR NMIGQGA		33
mouse-mRNA	VKWIFSSVVE LKQTLVPEYK NMIGQAP		34
mouse-Ig3	VKWIFSSVQ VKQTAIPDYR NMIGQGA		35
mouse-Ig3/2	VKWIFSSVQ VKQTAIPDYR NMIGQGA		36
rabbit	VKWIFSSVVE LKHTIAPDYR NMMGQGA		37
sheep-clone1/11	VKWIFSSV..		38

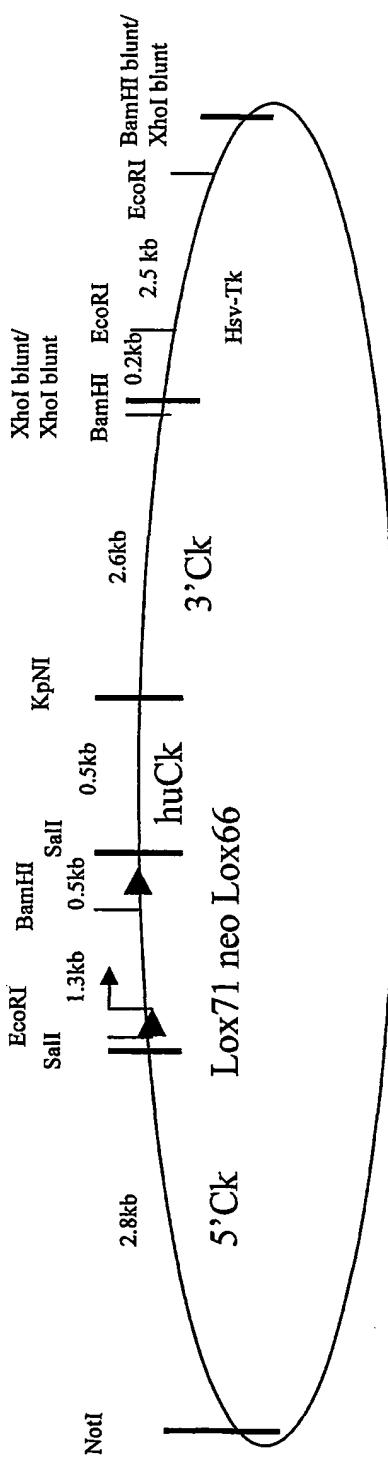


Figure 7a: DNA construct for the replacement of rabbit Ck with human Ck. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Cx1 gene. The upstream sequence (5' Ck) is 2.8 kb, the downstream sequence (3' Ck) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.

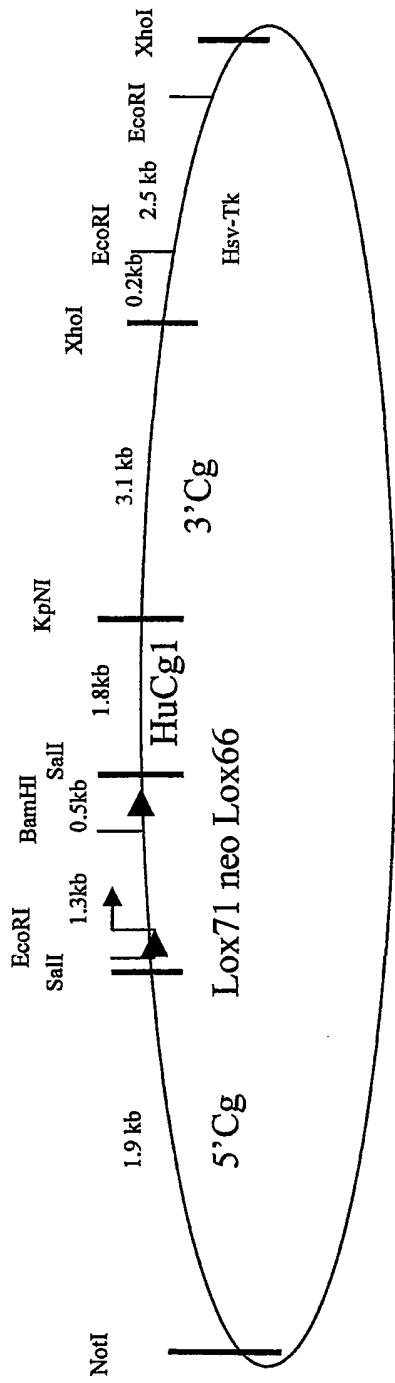


Figure 7b: DNA construct for the replacement of rabbit Cy1 with human Cy1. A 1.8 kb fragment containing a DNA sequence encoding human Cy1 is flanked by sequences from the rabbit Cy gene. The upstream sequence (5' Cy) is 1.9 kb, the downstream sequence (3' Cy) is 3.1 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cyl gene segment flanked by 50 nucleotides derived from the rabbit heavy chain immunoglobulin gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

aagggatcac atggcaccac tgacacct accctgccaa ggtcaggggt cctccaaggc
ctggcacccct cctccaagag caccctctggg ggcacagcgg ccctgggctg cctggtaag
gactactcc cccaaccgggt gacggtgtcg tggactca ggcgcctgac cagccgcgtg
cacacccctcc cggctgtccct acagtcctca ggactctact ccctcagcag cgtggtgacc
gtgccttcca gcaagcttggg caccaggacc tacatctgca acgtgaatca caagcccagc
aacaccaagg tgacaagaa agttggtagg agggcagcac agggagggag ggtgtctgt
ggaaggccagg ctcagcgctc ctgcctggac gcattccggc tatgcagccc cagtccagg
cagcaaggca ggcccccgtct gcctttcac ccggaggccct ctgcccggccc cactcatgt
caggagagg gtcttctggc tttttccca ggctctggc aggcacaggc taggtgcccc
taacccaggc cctgcacaca aaggggcagg tgctgggctc agacctgcca agagccatat
ccgggaggac cctgcccctg acctaaggcc accccaaagg ccaaactctc cactccctca
gctcgacac ctctctcttcc cccagattcc agtaactccc aatcttctct ctgcagagcc
caaatttgt gacaaaactc acacatgccc accgtgccc gtaagccag cccaggccctc
gcccctccagc tcaaggcggg acaggtgccc tagatagcc tgcatccagg gacaggcccc
agccgggtgc tgacacgtcc acctccatct cttcctcagc acctgaactc ctgggggac
cgtcagtctt cctcttcccc caaaaaccca aggacaccct catgatctcc cggaccctg
aggtcacatg cgtgggtgt gacgtgagcc acgaagaccc tgaggtcaag ttcaactgg
acgtggacgg cgtggaggtg cataatgcca agacaaagcc gggggaggag cagtacaaca
gcacgtaccg tgggtcagc gtccctcaccc tcctgcacca ggactggctg aatggcaagg
agtacaagtg caaggcttcc aacaaaggcc tcccagcccc catcgagaaa accatctcca
aagccaaagg tgggaccgg ggggtgcag ggccacatgg acagaggccg gctcggccca
ccctctgccc tgagagtgac cgctgtacca acctctgtcc ctacagggca gccccgagaa
ccacagggtg acacccctgccc cccatcccg gatgagctga ccaagaacca ggtcaggctg
acctgcctgg tcaaaggctt ctatcccagc gacatcgccg tggagtggg gagcaatggg
cagccggaga acaactacaa gaccacgctt cccgtgtctgg actccgacgg ctcccttcttc
ctctacagca agctcacccgt ggacaagagc aggtggcagc agggaaacgt ctctcatgc
tccgtgtatgc atgaggctt gcacaaccac tacacgcaga agagcccttc cctgtctccg
ggtaaatgag cgctgtgcc gcgagctgccc cctccctc ccccccacgc cgcagctgt.

Figure 9. The DNA fragment (SEQ ID NO: 52) containing a VH gene segment with more than 80% sequence identity with rabbit VH elements and encoding a human VH element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

tgagtgcacag tgtccctgacc atgtcgtctg tgtttgcagg tgtccagtgt
gaggtgcagc tgttggagtc cgggggaggt ctcgcccgac caggggggac cctgagactc
acctgcgcag tctctggatt cacccctca gtatgcacaa tgagctgggt ccgcaggct
ccaggaaagg ggctgaaatg ggtcggagcc attagtggta gtggtagcac atactacgcg
gacagcgtga aaggccgatt caccatctcc agagacaact ccaagaacac gctgtatctg
caaatacgtca gtctgagagc cgaggacacg gccccttatt actgtgcgaa agacacagtg
aggggccctc aggctgagcc cagacacaaa cctccctgca

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin light chain C_k gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

catccacat ggcacccagg
ggagatgtcc actggtaccc aagccctgccc atccctgtttg cttctttctt caggaactgt
ggctgcacca tctgtcttca tcttccccc atctgtatgag cagttgaaat ctggaaactgc
ctctgttgt tgcctgctga ataaattcta tcccagagag gccaaggat agtggaaagg
ggataacgc ctccaatcgg gtaaatccca ggagagtgtc acagagcagg acagcaagga
cagcacctac agccctcagca gcacccctgac gtcgagcaaa gcagactacg agaaaacacaa
agtctacgc tgcgaagtc cccatcagg cctgagctcg cccgtcacaa agagcttca
caggggagag tgttagagcg agacgcgtc cagggcaccc ccagcgcaccc tgaggccca
cctcgc.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a V_k gene segment with more than 80% sequence identity with rabbit V_k elements and encoding a human V_k element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

..ggcaggct gctcccaccc
catgcaggag gcagtaccag gcaggaccca gcatggacat gagggtccct gctcagctcc
tgggactcct gctgctctgg ctcccaaggta aggagggaaa caacaaaaat tttattcagc
cagttagcc actaatgcct ggcacttcag gaaattctt ttagaacatt actaatcatg
tggatatgt tttttatgtt cctaataatca gataccagat gttacatcca gatgacccag
tctccatcct ctctgtctgc atctgtggg gacagagtca ccatcactt ccgaggccagt
cagggcatta gcaattactt agcctggat cagcagaaac cagggaaagt tcccaagctc
ctgatttatg ctgcacatccac tttgcaatct ggggtcccat cgccgttca tggcagtgg
tctgggacag atttcactct taccatcagc agcctgcagc ctgaagatgt tgccacctat
tactgtcaaa agtacaacag tgccctcca ctttcggcg gagggaccaa ggtggagatc
aaacgtaagt gcactttcc aatgttcctc accgtttctg cctgatttgt ttgcttttc
catttttcgtat..

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides derived from the chicken light chain gene. The DNA sequence of chicken origin is underlined.

catacacag ccatacatac gcgtgtggcc gctctgcctc tctcttgcag gtcagccaa
ggctcccccc tcggtcactc tggtccggcc ctcctctgag gagcttcaag ccaacaaggc
cacactggtg tggctcataa gtgacttcta cccgggagcc gtgacagtgg cttggaaagc
agatagcagc cccgtcaagg cgggagttga gaccaccaca ccctccaaac aaagcaacaa
caagtacgcg gccagcagct atctgagcct gacgcctgag cagtggaaagt cccacagaag
ctacagctgc caggtcacgc atgaaggag caccgtggag aagacagtgg cccctacaga
atgttcatag tagtccact gggatgcaa tgtgaggaca gtggttcctc accctccctg

Figure 13. Modification of the chicken light chain locus using the ET system.
A chicken genomic BAC clone with the full length light chain locus was modified by homologous recombination. In a first step C λ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human C λ gene. The homology stretch was 50bp.

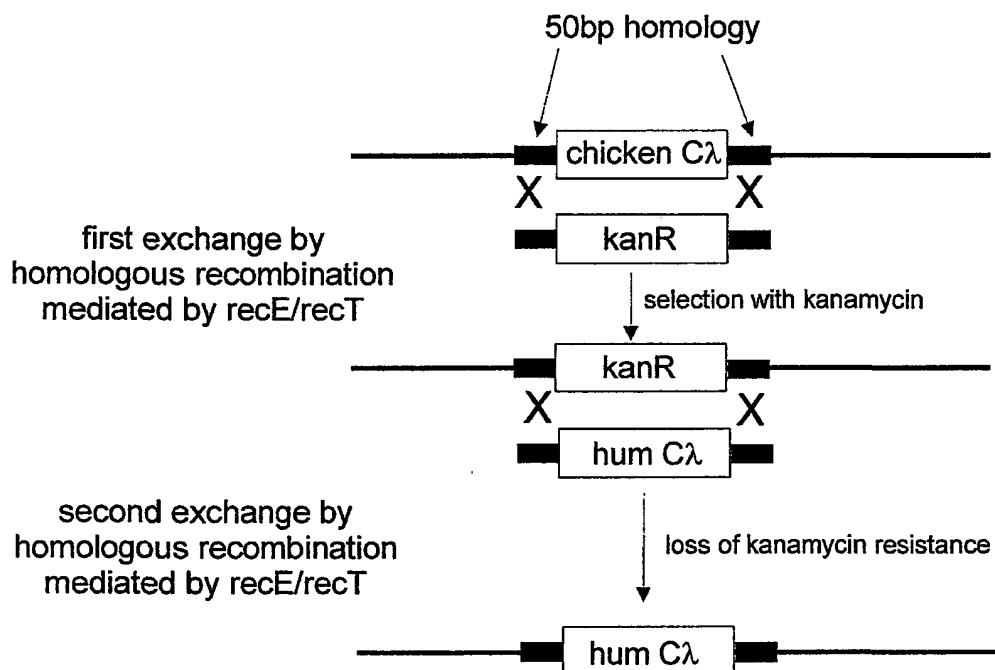


Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from chicken immunoglobulin DNA sequences are underlined.

.ttgccgttt tctccccctct ctcctctccc tctccagggtt ccctgggtgca gtcagtgcgt actcagccgc
cctcggtgtc agcagcccccg ggacaagaag tcacgatctc ctgctccggg tctagtagca acattggcga
taatttcgtc tcttgggtacC agcagctgcc tggcactgcc cctaagcttc tgatctatga taacaacAag
agaccctcggt gcatccctga ccgattctcc ggttccaaat ccggcacctc agccacatttta ggcatactgg
ggctccaaac cgccgacgag gctgactatt actgtgggac ttgggacagc agcctttctg ttggtatgtt
tggggcggg acacgcgtga ccgtccctagg tgagtcgtcg acctcgctc ggtctttctt ccccat...

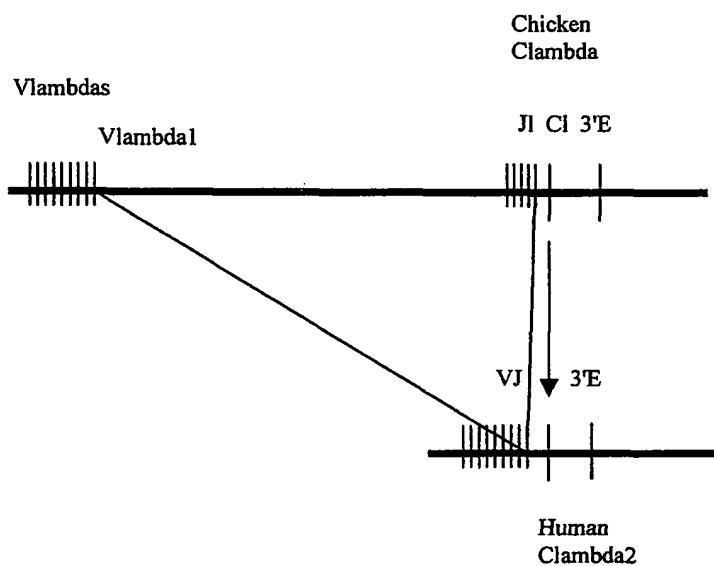


Figure 15. Humanized chicken light chain locus.

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(54) Title: PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) Abstract: This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/24348

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C12N 15/11,15/68,15/85;C07K 16/00;AO1K 67/00,67/083
US CL :586/28.1; 435/320.1,325; 580/387.1; 800/8, 18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 586/28.1; 435/320.1,325; 580/387.1; 800/8, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,075,181 A (KUCHERLAPATI et al.) 13 June 2000. see whole document.	1-66N
Y	DUVOISIN et al. Characterization of four constant region genes of rabbit immunoglobulin-lambda chains. J. Immunol. June 1986. Vol. 136. No. 11. pages 4297-4302, see whole document.	1-66
Y	SEHGAL et al. Generation of the primary antibody repertoire in rabbits: expression of a diverse set of Igk-V genes may compensate for limited combinatorial diversity of the heavy chain locus. Immunogen. 1999. Vol. 50. pages 31-42, see whole document.	1-66

Further documents are listed in the continuation of Box C. See patent family annex.

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"O"	document referring to an oral disclosure, use, exhibition or other means	"A"	document member of the same patent family
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 308-5230	Authorized officer Anne Marie BECKERLEG Telephone No. (703) 308-0196
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/24346

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MAGE et al. Evolutionary conservation of splice sites in sterile C mu transcripts and of immunoglobulin heavy chain (igh) enhancer regions sequences. Mol. Immunol. 1989. Vol. 26. No. 10. pages 1007-1010, see whole document.	1-66
Y	HEIDMANN et al. Molecular cloning of rabbit gamma heavy chain mRNA. Nuc. Acids Res. 1982. Vol. 10. No. 5. pages 1535-1545	1-66
Y	HAYZER et al. cDNA clones encoding immunoglobulin gamma chains from rabbit expressing the phenotype c7. Eur. J. Immunol. 1990. Vol. 20. pages 2707-2712, see whole document.	1-66

INTERNATIONAL SEARCH REPORT

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PCT/US01/24348

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG-Medline, Embase, Scisearch, Cancerlit, Biosis, BRS-EAST-USPATFULL, EPO, JPO, Derwent
search terms: xenomouse, humanized antibod?, transgenic, immunoglobulin, homologous recombination

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